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EFFECTS OF INTERACTIONS BETWEEN CHEMICALS AND NON-CHEMICAL STRESSORS ON THE GROUND BEETLE, *PTEROSTICHUS OBLONGOPUNCTATUS* (COLEOPTERA: CARABIDAE)

INTERAKCJE POMIĘDZY CHEMICZNYMI I NATURALNYMI CZYNNIKAMI STRESOWYMI U CHRZĄSZCZA *PTEROSTICHUS OBLONGOPUNCTATUS* (COLEOPTERA: CARABIDAE)

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„Nature is not only more complex than we think.
It is more complex than we can think."

Frank Edwin Egler 1970


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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Interactions between chemical and non-chemical stressors in invertebrates

Thanks to decades of studies of toxicity of various chemicals to a range of organisms, reasonably abundant and reliable data are available for dozens of organic and inorganic pollutants. However, more recently ecotoxicologists realized that pollutants only seldom are present in nature as single chemicals, and organisms are actually exposed often to mixtures of different chemicals. Consequently, it was suggested that good risk assessment methodologies should not relay on single-chemical ecotoxicological data but rather need to consider effects of mixtures of chemicals actually (or potentially) present in an area of interest (De Zwart and Posthuma, 2005); especially that interactions which may occur between chemicals may lead to various non-linear dose-response relationships for mixtures (cf., Jonker et al., 2005; Barata et al., 2006). Currently, two models are frequently used to predict mixture toxicity: similar action (SA; called also concentration addition, CA) and independent action (IA). SA model is based on the assumption that the mixture components have the same mechanism of action, i.e., they act on the same biochemical pathway additively. In contrast, IA model assumes that chemicals in the mixture work through different pathways and the probability of toxic effects caused by one chemical is independent from the probability of effects of another chemical. The accuracy of predictions generated by these models and different types of interactions (antagonism, synergism, dose-ratio and dose-level dependent deviations from the models) are currently debated (e.g., Martin et al., 2009). Some researches argue however, that for multi-chemical complex mixtures either SA or IA models can be used, offering sufficiently good approximation of a mixture toxicity. For example, Faust et al. (2003) showed that toxicity of a mixture of 16 different biocides could be predicted accurately with the independent action model. Moreover, differences between actual toxicity and that predicted by the concentration addition model never exceeded the factor of 3.2. In result, they concluded that "*With a regulatory perspective [...] concentration addition may be defendable as a pragmatic and precautionary default assumption*". If this is true, then it may appear that studies on toxicity of mixtures are not as crucial for risk assessment as originally supposed.

On the other hand, organisms, in their natural environment rarely experience optimal conditions, but they are forced to cope with sub-optimal and occasionally stressful environment, such as unfavorable temperatures, droughts, suboptimal availability of nutrients, periodic starvation, etc. Because specific combinations of these factors may cause effects that are greater or smaller than those of the pollutants acting at optimal conditions, there is some similarity to synergism (greater than additive toxicity) and antagonism (less than additive toxicity) in interactions between toxic chemicals. Nevertheless, those terms should be understood as purely phenomenological description of effects higher ("synergism") or lower ("antagonism") than would be expected under optimal environmental conditions. Although some authors suggest that IA model can be suitable for description of multiple stressor effect (Jensen et al., 2008; Long et al., 2009), this model does not relate to actual physiological and biochemical processes that stand behind interactions between natural factors and toxicants.

One of the most important natural factors, which is highly variable in the field and is of major importance for the physiological state of organism, is temperature. Due to different metabolic strategies, effects of temperature on toxic effects in poikilotherms and homeotherms may differ vastly. For example, poikilotherms, due to lower metabolism and activity at lower temperatures, may be less exposed to chemicals. The metabolic strategy of homeotherms is completely different – with temperature decreasing below an optimal range, their metabolic rates increase in order to maintain constant body temperature. Increasing metabolic rate requires increased consumption, and if food is contaminated with toxic chemicals this may lead to increase assimilation of the chemicals. Temperature affects also environmental fate of many contaminants (e.g., adsorption, degradation) and hence exposure of organisms to them. Temperature should be therefore taken into consideration when assessing effects of anthropogenic pollutants on organisms. Surprisingly, the occurrence of interactions between temperature and environmental toxicants, such as metals or pesticides, has not received much attention in ecotoxicological studies, although the relevance of this problem was pointed out already 25 years ago by Bryant et al. (1985) and Demon and Eijsackers (1985). Only recently the number of papers on interactions between temperature and

chemical factors in terrestrial invertebrates increased substantially. For example, a highly significant effect of interaction between Hg and low and high temperatures was observed on survival of the springtail, *Folsomia candida* (Holmstrup et al., 2008; Slotsbo et al., 2009). On the other hand, Jensen et al. (2008) found synergistic interactions (i.e., the observed effect was higher than at non-stressing temperature) between nonylphenol and high but not low temperatures in the earthworm *Dendrobaena octaedra*. The authors suggested that nonylphenol might disrupt membrane stability during thermal stress and/or deplete protective HSP action. High temperature also increased the toxicity of chlorpyrifos in the moths *Earias vitella*, probably by influencing the physical and chemical state of the pesticide and, thereby, accelerating its biotransformation into more toxic metabolites (Satpute et al., 2007).

The interactions of three (or more) stressors are rarely studied (Chen et al., 2004; 2008; Heugens et al., 2006), mostly due elaborate experimental design and complex interpretation of higher order interactions. No data are available on combined effects of chemicals with different modes of action and natural factors for terrestrial invertebrates.

The aim of this thesis was to obtain more insight into combined effects of metals, pesticides and temperature on terrestrial invertebrates. Unique is the attempt to link information on effects of multiple stresses with mechanisms of metal uptake and elimination under the influence of additional chemical or non-chemical factors.

Throughout the thesis the term "natural stressors" is used not only for natural environmental factors at highly stressing levels, but also at suboptimal levels. From the point of view of organisms' physiology and fitness, all suboptimal conditions are stressful to a certain degree, and for ecological risk assessment a more general information about effects of suboptimal conditions is interesting and needed. Therefore the term "natural stressor(s)" is used herein interchangeably with "natural factor(s)".

Strategies of detoxification of chemicals in terrestrial invertebrates

In this section, mechanisms of pesticides and metals detoxification are presented briefly, with special focus on key differences between organic pollutants and metals; detailed description of different strategies is beyond the framework of this research.

Any chemical has to be first taken up by an organisms to cause a toxic effect. Once a chemical is taken up into the body, it can be stored, metabolized, or bound to a receptor and thus cause a toxic effect. In case of organic pollutants, not only the parent compound can cause toxicity but also metabolites. For example, chlorpyrifos (CPF), a broad spectrum systemic phosphorothioate ester, may be transformed inside organisms to more toxic chlorpyrifos-O-analaog. Biochemical systems responsible for toxicity of CPF include cytochrome-P450-mediated activation of this insecticide to the oxon metabolites (desulfuration), target enzyme sensitivity to the chlorpyrifos-oxon, and detoxification of the OPs insecticides and their oxons. Detoxification routes include dearylation of the phosphorothionates by P450, aliesterase phosphorylation by the oxons, and A-esterase-mediated hydrolysis of the oxons.

In contrast to organic pollutants, metals cannot be metabolized. Some species may limit bioaccumulation of certain metals either by reducing their uptake or by active excretion, thereby maintaining low metal body burdens even at high exposure concentrations. Other species, however, are not able to regulate bioaccumulation of non-essential metals, but prevent toxicity by effectively storing metals in non-toxic forms, for example bound to metal-binding proteins like metallothionein (MT) or incorporated into non-soluble granules (Vijver et al., 2004), and excreting thus stored (detoxified) metal. Metals such as Cd, Cu, and Pb mainly have affinity to nitrogen- or sulfur-containing groups, whereas other metals (e.g. Ca, Al) bind more effectively with oxygen-containing groups, such as carboxylic acids. Metals from the third group, which includes Zn and Ni, have no binding preference and form ligands with many functional groups (Nieboer and Richardson, 1980). Species relying on effective storage of metals in an inert form, which cannot or only slowly can be eliminated from the body, accumulate metals excessively with increasing exposure concentrations without

suffering toxic effects. Such strategy, which was found, for example, in spiders (Janssen et al., 1991), results in a linear uptake pattern. Adverse effects may occur, however, when the capacity of the detoxification mechanism is exceeded. Therefore, many organisms (e.g. carabid beetles, earthworms) evolved some ways of decontamination/depuration allowing to excrete at least part of the assimilated toxicant (Janssen et al., 1991; Kramarz, 1999a; Vijver et al., 2005), and such strategy of metals detoxification results in a saturation-type uptake curve.

Modelling metal turnover through organism

There is a growing body of data indicating that bioavailability of metals (i.e. the fraction of a metal that is available or can be made available for uptake and, as a consequence, can cause toxic effects in organisms) may be estimated from assimilation rates (e.g. Van Straalen et al., 2005; Luoma and Rainbow, 2005). Estimation of assimilation rate in the presence of simultaneous elimination is improved significantly if the uptake is followed by an elimination phase without uptake (Van Straalen et al., 2005). Therefore, in toxicokinetic experiments an uptake phase (contamination phase) is usually followed by an elimination phase (decontamination phase), in which organisms are transferred to a clean medium and food after a certain period of exposure to a metal. The turnover of metals through an organism is often calculated using one-compartment models, although a two-compartment model was required, for example, to describe zinc and cadmium kinetics in earthworms (Vijver et al., 2005).

Metal uptake, distribution, tissue accumulation and excretion depend on many factors, including physiological properties of a metal, routes of exposure and physiological attributes of organisms (Depledge and Rainbow, 1990), and may differ for different organisms (Hames and Hopkin, 1990; Janssen et al., 1991). Some authors have stressed that uptake and elimination kinetics also depend on external factors such as temperature (e.g., Spurgeon et al., 1997; Tessier et al., 1994; Van Hattum et al., 1991), but the experimental studies on this subject for terrestrial invertebrates are limited (Janssen and Bergema, 1991).

Chemical stressors: a metal (nickel) and a pesticide (chlorpyrifos)

Nickel (Ni) contamination originates mostly from industry, including mining, stainless steel production, and battery manufacturing. These activities have resulted in the redistribution of significant amounts of Ni in the environment (Kabata-Pendias, 2000). Limited information is available on essentiality of Ni or homeostatic mechanisms that regulate Ni accumulation and elimination in soil invertebrates (Eisler, 1998). Existing research has been limited mainly to comparing Ni concentrations in the external environment (i.e., soil, foliage food) to body residue concentrations in various invertebrate species (Bagatto and Shorthouse, 1996; Janssen et al., 1997; Kozlov et al., 2000; Neuhauser et al., 1995; Peijnenburg et al., 1999), including insects adapted to the environment rich in Ni (reviewed by Boyd, 2009). Experiments on nickel uptake and/or elimination kinetics concentrated on aquatic species (Gills et al., 2004; Klerks and Fraleigh, 1997). As regards Ni mode of action, microarray analysis of Ni-exposed *Daphnia magna* revealed recently several affected functional gene classes involved in different metabolic processes (mainly protein and chitin related processes), cuticula turnover, transport and signal transduction (Vandenbrouck et al., 2009). Transcription of genes involved in oxygen transport and heme metabolism was down-regulated in organisms exposed to elevated Ni concentrations.

Relatively more is known about the second chemical examined in the present study – chlorpyrifos (CPF). Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] was first described in 1965 as a useful insecticide for a wide range of insect pest species and to date it is used for controlling agricultural, horticultural and forest pests. Chlorpyrifos is toxic to animals through inhibition of an enzyme acetylcholineserase (AChE), thereby disrupting cholinergic nerve transmission (Stenersen, 2004). It was shown, for example, that AChE activity was reduced by ca. 60% in the wolf spider, *Lycosa hilaris* exposed to soil spiked with 2-4 mg kg^{-1} CPF (Van Erp et al., 2002). Also, some examples of the joint effect of other organophosphorous pesticides (OPs) and metals on AChE as well as detoxifying and antioxidative enzymes are known for terrestrial invertebrates (Babczyńska et al., 2006; Augustyniak et al., 2005), but a few studies on joint effects of Ni and OPs on terrestrial

invertebrates have been published (Augustyniak et al., 2007; Zawisza-Raszka and Dolezych, 2008).

Test organism

To study the multiple stressor effects on terrestrial invertebrates, the ground beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae) was used as a model organism. *P. oblongopunctatus* is a relevant study object in this respect because it is not unusual that in their natural ecosystems beetles may suffer from exposure to metal pollution originating from industry and from the use of pesticides in forestry and agriculture. Moreover, because they are widely distributed in Palearctic (Brunsting 1981), where high seasonal and diurnal temperature fluctuations are the norm, they are certainly subjected occasionally to suboptimal temperatures; of course also in polluted areas. This species received already wide interest in research on effects of environmental changes, such as fragmentation and pollution (Łagisz et al., 2002; Migula et al., 2004), as well as those focused on effects of chronic, multigenerational exposure to metals and resistance to toxic metal concentrations (Mozdzer et al., 2003; Lagisz et al., 2005; Lagisz and Laskowski, 2008). The ability of *P. oblongopunctatus* to survive in metal polluted areas is well known, but it was shown that toxic metals can affect their physiology (Lagisz et al., 2002; Stone et al., 2002) and susceptibility to additional stressors (Stone et al., 2001). Thus, effects of exposure to metals can be magnified in field-living carabids by other chemical and/or natural stressors.

Aims of the study

In this thesis the interactions between chemicals with different modes of action and between chemicals and temperature were tested. It was also assumed that natural stressors can modify effects of interactions between chemicals. These hypotheses were tested using data generated in laboratory experiments on the ground beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae). Nickel and chlorpyrifos were used as examples of a widespread pollutants with different mode of action, namely metals and pesticides, and temperature was used as a natural factor.

First, range-finding experiments were performed with single stressors to determine the susceptibility of *P. oblongopunctatus* to nickel, chlorpyrifos and temperature. The results from experiments on Ni toxicity to the beetles and effect of Ni and temperature on the beetles respiration rates are described in Chapter 2.

Combined effects of environmental pollutants (Ni, CPF) and temperature on beetles were studied in a full-factorial design with at least three levels of each factor. The recorded endpoints for adult beetles were lifetime and reproduction rate. The results are reported in Chapter 3. The effects of the same stressors on soil-dwelling larvae were quantified in terms of mortality and proportion of emerged imagines. Results are presented in Chapter 4.

The next section of this thesis (Chapter 5) addresses toxicity mechanisms underlying the interactions between the studied factors. For this purpose Ni assimilation and elimination rate constants in the beetles exposed to contaminated food either at different temperatures or under the influence of CPF were derived based on the one-compartment model. Because the mechanism of Ni bioaccumulation appeared to be more complicated than described by the classic one-compartment model, modified toxicokinetic models were proposed to describe the behaviour of this metal in *P. oblongopunctatus*. The advantages of using alternative models, especially the one allowing for different assimilation/elimination rates in different phases of exposure to Ni, are discussed in Chapter 5.

Finally, the summary provides a general discussion of results obtained in all experiments. An overview of the main findings is given, and the research is discussed in a more general context of ecological risk assessment.

CHAPTER 2

EFFECTS OF NICKEL AND TEMPERATURE ON THE GROUND BEETLE *PTEROSTICHUS OBLONGOPUNCTATUS* (COLEOPTERA: CARABIDAE)

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Abstract

In natural ecosystems it is not unusual for an organism to be exposed both to chemical and physical stressful factors at the same time. Herein results of the study on nickel (Ni) toxicity to the carabid beetle, *Pterostichus oblongopunctatus*, and effect of Ni and temperature on the beetles respiration rates are presented. In the first part of the study (Experiment I) the survival, respiration rates and internal Ni concentrations were measured in animals exposed for 245 d at constant temperature (20°C) to food contaminated with Ni at nominal concentrations 0, 600, 1200, 2400, 4800, and 9600 mg kg^{-1} dry weigh (dw). The LC_{50} was estimated at 8351 mg Ni kg^{-1}, with no effect on fertility. A significant positive correlation between Ni concentration in food and internal body concentration of Ni, and a negative correlation between Ni exposure and the respiration rate were found. Based on these results, the concentration of 2400 mg kg^{-1} (LOEC for the respiration rate) was selected for the second part of the study (Experiment II) in which field-collected males of *P. oblongopunctatus* were exposed to Ni-contaminated food for 64 d and then to uncontaminated food for the next 64 d at three temperatures: 10°C, 15°C and 20°C. In this part of the study it was found that the temperature under which the beetles were kept affected their respiration rates, and that effect of Ni on the respiration was significant only in animals originating from 20°C. The results from both experiments indicate that negative effects of nickel appear only after relatively long exposure.

Keywords: nickel, temperature, respiration rate, ground beetles

Introduction

Virtually all organisms are exposed to multiple natural environmental stressors, such as events of unfavourable temperatures, suboptimal availability of nutrients, periodic starvation, or inadequate levels of moisture. A few studies showed already that these stressors can indeed affect organisms responses to toxicants (cf. Abdel-Lateif et al., 1998; Holmstrup et al., 2000; Bednarska et al., 2006), however, the subject is rather poorly studied, especially within a risk assessment context, or even within ecotoxicology in general. This consideration served as the impetus for this study, which focuses on the effects of temperature on the toxicity of nickel (Ni) to the carabid beetle *Pterostichus oblongopunctatus*.

Nickel is a naturally occurring element, but anthropogenic sources are responsible for its elevated concentrations in the environment. Since industrialization, large amounts of Ni have been released to the environment, especially from burning fossil fuels. Nickel is usually emitted from smelters as very fine dust particles which remain in the atmosphere for a long time and can be transported to long distances. In the vicinity of smelters, Ni concentrations in soil and plants may exceed its natural content 100 times (Eisler, 1998; Kabata-Pendias, 2000) or even more, since in smelter-contaminated soils concentrations as high as 22,000 mg kg^{-1} may occur (Everhart et al., 2006). Since six different Ni-containing enzymes have been identified (Ermler et al., 1998), the biological importance of Ni is unquestionable. Nickel can replace other metal ions in enzymes and proteins and has the ability to bind to cellular compounds containing O^{-2}, S^{-2}, and N^{-} ions and inhibit their functioning.

Increasing Ni output and processing in the recent years have resulted in increased interest in its environmental fate and ecotoxicology among scientist. Toxic effects of Ni were studied in soil invertebrates such as earthworms (Scott-Fordsmand et al., 1998; Lock and Janssen, 2002) and springtails (Scott-Fordsmand et al., 1999), where test organisms were exposed to increasing concentrations of the metal in soil under constant ambient conditions in laboratory. However, in the field natural stressing factors are likely to modify responses of animals to chemical exposure through their

influence on a variety of physiological processes. For example high temperature, by increasing metabolic rates, can increase consumption and assimilation of toxicants contained in food and, thus, may lead to increased intoxication of exposed animals. On the other hand, the elevated metabolic rate at high temperatures may help to increase rates of detoxification and elimination of toxicants from an organism.

Respiratory metabolism is one of the main components of an energy budget and, at the same time, one of the most sensitive to both internal and external factors (e.g., body mass, sex, physiological state, circadian rhythm, temperature) (Chaabane et al., 1999; Rowe et al., 2001; Łagisz and Laskowski, 2002). It is also relatively easy to measure. As such it is often used as the equivalent for a metabolic rate (Migula, 1989; Handy and Depledge, 1999). Thus, it can serve as a convenient end-point in ecotoxicological studies in which effects of toxicants and other factors on metabolic rate may be expected.

Herein results of the study on effects of interaction between temperature and Ni exposure on the ground beetle *P. oblongopunctatus* (Coleoptera: Carabidae) are presented. Carabid beetles are for many reasons particularly interesting for ecotoxicology: being important pest-control species they need special attention in environmental risk assessment, they are relatively abundant in most terrestrial ecosystems and can be easily collected for both field and laboratory studies. They are poor accumulators of metals (Laskowski and Maryański, 1993), which may results from efficient mechanisms of detoxification and excretion (Janssen, 1991; Kramarz, 1999a), but individuals inhabiting chronically polluted environments appear less tolerant to additional environmental stressors then those from uncontaminated areas (Stone et al., 2001). Thus, effects of exposure to metals can be magnified in field-living carabids by other chemical and/or natural stressors.

In this study the life-cycle traits (survival and reproduction) and respiration rates of adult beetles were measured in controlled laboratory conditions, simulating exposure to Ni at different temperatures. The rationale for using such endpoints is that a response to intoxication is costly for an organism in terms of metabolic demand (which is

reflected in increased respiration rate), and this additional energy expenditure diminishes the resources available for reproduction and/or maintenance (Sibly and Calow, 1989). It was also hypothesized that effects of Ni exposure should be temperature dependent because higher metabolic rate at higher temperatures should result in increased food demand and, thus, higher metal assimilation. On the other hand, the increased metabolic rate may allow for more efficient detoxification. Thus, the relationship between metal exposure, temperature and toxic effects seems complex, and the net result is hard to predict.

Materials and Methods

Two experiments were performed to characterize effects of Ni and temperature stress on beetles (*P. oblongopunctatus*). The purpose of Experiment I was to provide information about Ni body levels and effects on the measured endpoints (survival, reproduction and respiration) resulting from a long-term Ni exposures at a broad range of concentrations in food. In Experiment II, dietary Ni was kept either at "zero" or at high level for 64 d, and the animals were reared at 10, 15 or 20°C. Then the animals were allowed to depurate for another 64 d. In this experiment, respiration rates were measured three times during the Ni-contamination period, and again at the end of the study. Detailed description of the two experiments is given below.

Experiment I

The laboratory culture of *P. oblongopunctatus* was established from appoximately 120 adult beetles collected with pitfall traps at uncontaminated site near Krakow, southern Poland. Metal concentrations in the humus layer where the beetles were captured were (mg kg^{-1} dry weight): Zn, 108.7; Pb, 156.5; Cd, 1.5; Ni, 5.2 at pH_{H_20} 4.5 (Stefanowicz at al., 2008). The beetles were kept in a climate-controlled chamber at 20°C, under a light:dark regime (L:D) of 16:8 h, with relative humidity (RH) at 75% and the light intensity of 500 lx. The beetles were kept in 1000-ml plastic boxes, 5 pairs per box; the boxes had perforated lids and contained about 2 cm of moist peat at pH_{H_2O} 4.5-5.0 and 80% of its water-holding capacity (WHC). Every third day, eggs were collected with forceps and deposited individually on moist filter paper in 24-well tissue culture plates.

The plates were stored at 20°C in darkness and controlled daily for newly hatched larvae. The larvae were transferred individually to 30-ml plastic vials filled to approximately 3/4 with moistened peat with a 3 cm deep hole made with a needle. The larvae were cultured at summer conditions (16:8 h L:D, 20°C, 75% RH, 500 lx) until imago.

The animals were fed *ad libitum* with artificial food made of ground mealworms mixed with ground apple (7:3 dry weight) with 1 g sodium benzoate ($C_7H_5NaO_2$; Fluka, Deisenhofen, Germany) per kg food as preservative.

Twenty-eight days after emergence, the adults were sexed and five males and five females were randomly assigned to one of the six experimental treatments (0, 600, 1200, 2400, 4800, 9600 mg Ni kg^{-1} dry food). Nickel chloride ($NiCl_2 \times 6H_2O$; Eurochem BGD, Warsaw, Poland) was added to the food as aqueous solutions. For the next 208 days the beetles were kept individually in plastic vials filled to approximately 3/4 with moistened peat. After 30 days of exposure in summer conditions, the beetles were cultured as shown on Fig. 2.1. They were transferred to autumn and then to winter conditions for 12 weeks, and after overwintering all beetles were transferred to spring conditions and then back to summer conditions. Thus, the conditions during the experiment simulated natural seasonal changes. The animals were fed *ad libitum* every third day during 'spring', 'autumn' and 'summer', and twice a week during 'winter'. When fresh food was supplied, the remains of old food were taken out to keep the boxes and the vials as clean as possible.

After 192 d since the start of the experiment, respiration rates of the beetles were measured for 68 h. Two weeks after the respiration measurements the animals were coupled (a male and a female from the same treatment), and each pair was transferred to a separate box. The recorded endpoints were survival and the reproduction rate expressed as the number of eggs produced per female. The experiment ended after 245 d. At the end of the experiment, the carabids were starved for 24 h in order to void their guts, and then were frozen for metal analysis. Animals which died in the course of the experiment were excluded from the metal analysis.

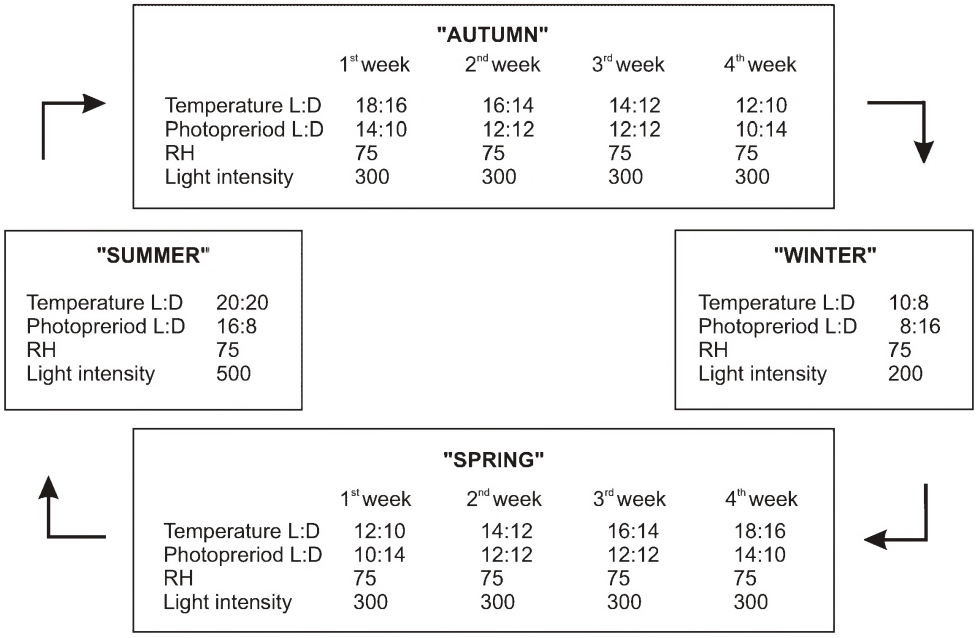

Fig. 2.1. A scheme of the experimental conditions of *Pterostichus oblongopunctatus* culture in the Experiment I; L:D - light:dark regime (h), RH – relative humidity (%), light intensity (lx).

Experiment II

Based on results of the Experiment I, the concentration of 2400 mg Ni kg^{-1} dry food was selected for this part of the study. At this concentration the beetles could survive for a long time and reproduce, but their respiration rates were significantly decreased, indicating some negative effects of Ni on metabolism (see Results).

Before starting the experiment, adult males of *P. oblongopunctatus* collected in April 2006 from the same site as for the Experiment I were kept for 2 weeks individually in 30-ml plastic vials filled to approximately 3/4 with moist peat (pH_{H_2O} 4.5 - 5.0, 80% WHC) at constant temperature (20°C), photoperiod 16:8 h L:D, 75% RH and light intensity of 500 lx. During this acclimation period the carabids were fed *ad libitum* with uncontaminated artificial food (same as in the Experiment I) every 3rd day. Then, the animals were randomly allocated to three experimental groups differing only in ambient temperature (10, 15 or 20°C). The temperatures were chosen based on the results from an earlier range-finder study, in which it was found that 20°C is optimal

for survival and reproduction, at 25°C the beetles almost completely stop reproducing, and at 30°C they do not survive long enough to study long-term effects (LT_{50} was 11 d). After one week of acclimation to experimental temperatures, the animals from each temperature group were randomly assigned to control or Ni-treatment group. The animals were then fed for the next 64 d with either clean food (control) or contaminated with Ni at 2400 mg kg^{-1} (contamination phase). For another 64 d, all beetles were fed uncontaminated food (decontamination phase). In all treatments respiration rates of the beetles were measured on days 16, 32, 64 (contamination phase), and 128 (the end of the decontamination phase) since the beginning of the experiment (Fig. 2.2).

Respiration rate measurements

After being weighed to the nearest 1 mg (WPA 180/k Radwag, Radom, Poland), the animals were placed individually into 50-ml flasks connected to a 30-channel Micro-Oxymax respirometer (Columbus Instruments, USA). The animals from different treatments were assigned to the flasks at random. A punctured Eppendorf-type tube filled with distilled water was placed in each flask to prevent desiccation. The respiration rate was measured at 4-h intervals for 68 h in Experiment I, and for 76 h in Experiment II. The animals were not fed during the measurements. Because of the limited number of respirometer channels, the measurements were taken in two series. In each series, five animals per Ni concentration were used in the Experiment I, and five animals per concentration per temperature in the Experiment II. Thus, in each experiment respiration rate was measured in 10 animals per treatment. To avoid confounding effect of temperature during respiration measurements (in poikilotherms the respiration rate increases with temperature exponentially), all measurements were performed at 20°C. To minimize the problems arising potentially form the fact that the beetles from lower temperatures needed some time to acclimate to 20°C, the beetles were transferred to this temperature about 4 h before starting the respiration measurements. Additionally, prior to data analysis, the first measurement point (the first 4-h interval) for each individual was excluded from the data, since it was suspected that not only temperature but also the change of the environment and

handling stress might cause temporarily abnormal activity and respiration rates. After the measurements the beetles were returned to their original rearing conditions (Fig. 2.2). Animals which died in the course of respiration rate measurements were excluded from statistical analysis.

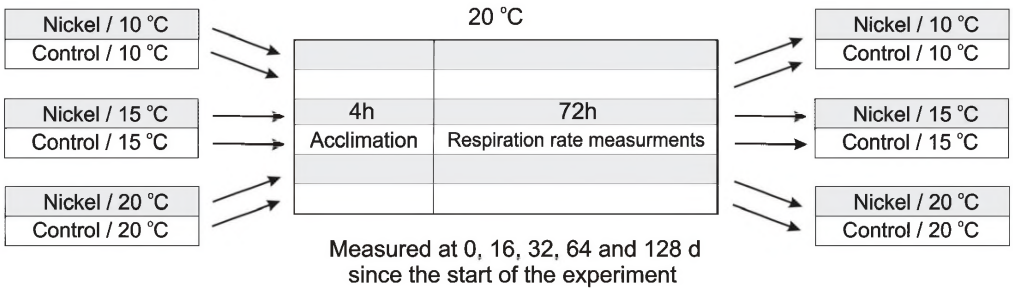

Fig. 2.2. A scheme of the Experiment II design.

Respiration rate was measured as oxygen consumption per hour per beetle, recalculated per gram body mass for data analysis, and expressed in µl O_2 g^{-1} h^{-1}.

Chemical analysis

For metal analysis the frozen beetles were dried at 105°C for 24 h. After weighing to the nearest 1 mg (WPA 180/k Radwag, Radom, Poland), the animals were digested in 2 ml boiling nitric acid (suprapur-grade HNO_3; Merck, Darmstadt, Germany). During the procedure, the temperature was raised gradually from room temperature to 150°C, and when a sample was mineralized, the excess of HNO_3 was evaporated to the volume below approximately 0.05 ml, and the sample was diluted to 5 ml with deionized water. Samples of food were prepared for analyses in a similar way: approximately 0.1 g dry sample of each batch was mineralized in 2 ml boiling HNO_3 and diluted to 10 ml. Nickel concentrations in the beetles and in food were determined by atomic absorption spectrophotometer (AAnalyst 800, Perkin-Elmer, Boston, MA, USA). Nickel in the beetles was analyzed in a graphite furnace (detection limit: 0.56 µg L^{-1}), while Ni in food was measured with a flame method (detection limit: 0.13 mg L^{-1}). To calculate the detection limits, 10 calibration blank samples were analyzed. The

detection limits were calculated as three standard deviations for the mean of the calibration blank measurements and were at least five times lower than the lowest concentration measured in analytical samples. Blank samples were run with each analytical batch to control the method for possible contamination, and samples of standard reference material (dogfish muscle DORM-2; National Research Council, Ottawa, ON, Canada) were used to check analytical precision: the measured concentrations were within ± 9 % of the certified reference value. Nickel concentration in the beetles and in the food was expressed in mg kg^{-1} dry weight.

Statistical analysis

The distributions of the data were checked for normality with Shapiro-Wilk's W test. Because most of the data were not normally distributed, they were log-transformed and this satisfied the criterion in all cases but the respiration rates. The respiration rates were rank-transformed prior to ANOVA as in this case the variances differed among treatments. Rank transformation was chosen because the variance of rank data is automatically stable (Potvin and Roff, 1993). Outliers with the absolute values of modified MAD z-score greater than 3.5 were excluded from ANOVA.

In the Experiment I multiple regression was used to find out which variables affected the endpoints measured. The independent variables were: sex, body weight, and Ni concentration. The variables with the highest p value were removed consecutively from the model (downward stepwise procedure with cut-off value F=4.0) as long there were any variables with p>0.05. The non-linear regression was used to estimate LC_{50}. The model allowing for hormesis was used (Løkke, 1995):

$$y = \frac{c + hx}{1 + (c/2 + hLC_{50})/(LC_{50}^{b}/2)x^{b}}$$

where parameter h allows for a stimulatory effect at low concentrations (hormetic effect), c is the survival in control treatment (in this model always set to 100%), x the concentration of Ni in food, and b the shape parameter. The parameters were estimated with the Gauss-Newton method.

For treatments in which mortality during the experiment was large enough, the LT_{50} (the median survival time) was estimated from survival analysis (Kaplan and Meier, 1958). If significant treatment effect was found ($p<0.05$), a comparison against the control was performed, with the log-rank test, in order to detect which concentrations caused significant reduction of life time.

In the Experiment I rank respiration rates were analyzed using two-way ANOVA, with treatment (Ni concentration in food) and sex as explanatory factors. Nonsignificant variables were removed from the model, and means were separated with LSD test.

In the experiment on interaction between temperature and Ni toxicity (Experiment II), rank respiration rates were compared between Ni treatments (i.e., 0 or 2400 mg kg^{-1}), temperatures, series and days of the experiment using multifactor ANOVA with wet body mass as the covariate. The interactions and variables with the highest p value were removed consecutively from the model as long there were any interactions/variables with $p>0.05$.

All analyses were done using Statgraphics Centurion XV (Statpoint Inc.; www.statgraphics.com) and CSS-Statistica programs.

Results

The actual Ni concentrations in food were in good accordance with assumed nominal values (Table 2.1).

Two animals which escaped before the end of the experiment were excluded from LC_{50} analysis, but incomplete (censored) life data for those animals were included in survival analysis. One outlier among 53 values was excluded from two-way ANOVA in the Experiment I, and eight outliers among 222 values were identified and excluded from multifactor ANOVA in the Experiment II.

Table 2.1. Nominal and measured nickel concentrations in food for *Pterostichus oblongopunctatus* and whole body nickel concentrations in beetles at the end of the Experiment I (mean ± SD). Sample sizes (number of food batches and number of beetles analyzed) are given in brackets.

Nominal Ni concentration (mg kg^{-1})	Measured nickel concentration in food (mg kg^{-1})	Nickel concentration in beetles (mg kg^{-1})
0	10.6 ± 4.2 (3)	2.6 ± 1.2 (7)
600	612 ± 54.8 (5)	26.3 ± 17.9 (8)
1200	1145 ± 364 (5)	59.0 ± 35.9 (9)
2400	2425 ± 161 (4)	164 ± 139 (9)
4800	5036 ± 405 (5)	127 ± 82.2 (7)
9600	10,094 ± 402 (5)	725 ± 405 (2)

Experiment I

Males were significantly lighter than females (p=0.004), but no significant effect of Ni on body mass was found (p>0.47). The mean body mass from all treatments (including control) was 44.5 mg for females, and 41.0 mg for males.

Only five of ten beetles from 9600 mg Ni kg^{-1} survived until the day of respiration rate measurements, and at the end of the experiment only two beetles were still alive. The LT_{50} for the beetles fed 9600 mg kg^{-1} was 192 ± 22.1 d. In the other treatments the mortality did not exceed 30% (three beetles died in the control, two in 600 mg Ni kg^{-1} and one in 4800 mg Ni kg^{-1}) meaning that LT_{50}s were higher then 245 d and were thus not estimated. A pair-wise comparison revealed that only the life time of the beetles from the highest Ni treatment differed significantly from the control (p=0.004).

The regression model relating mortality to Ni concentration was well fit (p=0.0004; R^2=96.5%), and the LC_{50} value was estimated at 8351 mg Ni kg^{-1} (asymptotic 95% confidence interval: 5911-10792; p=0.002) (Fig. 2.3). At lower concentrations significant hormesis was found (h=0.025; p=0.016), and the shape parameter b was estimated at 3.74 (p=0.01). Due to the very high inter-individual variability in the number of eggs produced per female, no significant effect of Ni on beetles

reproduction (p>0.15) was found. However, at the highest Ni concentration at which females survived until coupling (4800 mg kg^{-1}) only one of five females reproduced, and in all treatments but control and 1200 mg kg^{-1} there was at least one non-reproducing female.

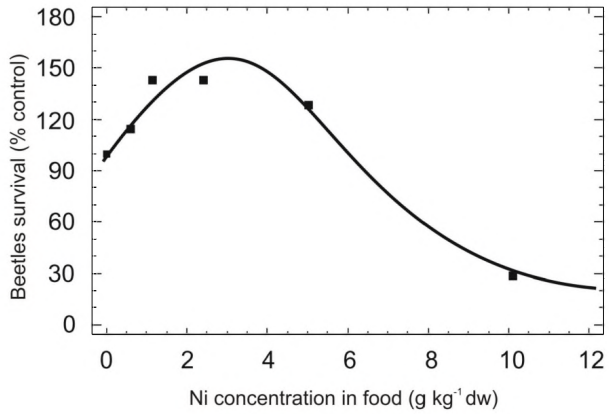

Fig. 2.3. Effect of nickel concentration in food on survival of *Pterostichus oblongopunctatus* in the Experiment I; p=0.0004, R^2=0.97; note the significant hormesis (p=0.002).

Body Ni concentration increased significantly with exposure (p<0.0001) with no differences between sexes (p>0.13). The regression model explained 73% of total variability in Ni concentration in *P. oblongopunctatus* (Fig. 2.4).

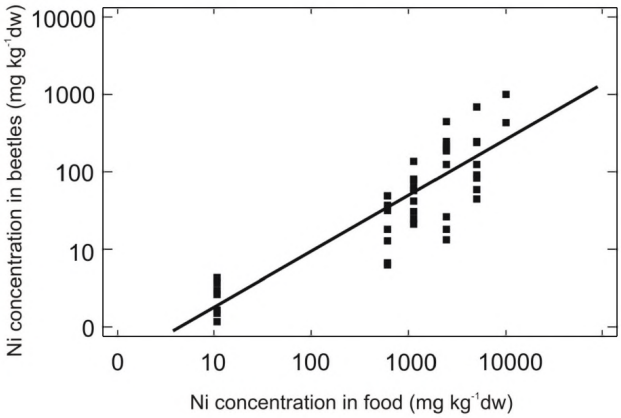

Fig. 2.4. Effect of nickel concentration in food on internal body concentration of nickel in *Pterostichus oblongopunctatus* at the end of the Experiment I; p<0.0001, R^2=0.73.

The average whole-body Ni concentration at 4800 mg kg^{-1} was about 50-fold higher than in control beetles, and at 9600 mg kg^{-1} almost 280-fold higher (Table 2.2). However, the latter value is based on only 2 beetles that survived until the end of the experiment.

Table 2.2. Nominal treatments (nickel concentrations in food) and respiration rates in *Pterostichus oblongopunctatus* measured at 192 d of the Experiment I (mean ± SD).

Nominal treatment (mg kg^{-1})	Number of beetles analyzed *	Respiration rates of beetles (µl O_2 g^{-1} h^{-1})
0	10	332 ± 96.5 **A****
600	10	295 ±77.0 **A**
1200	8	256 ± 25.0 **AB**
2400	9	241 ± 31.6 **B**
4800	10	248 ± 38.5 **B**
9600	5	267 ± 71.2 **C**

* results obtained for both sexes were compiled because no significant difference in respiration rates between the sexes was found by two-way ANOVA;
** the same letter means no significant differences in respiration rates between treatments (p>0.05).

The respiration rate decreased with increasing Ni concentration (p<0.0001). The model included actual Ni concentration in food (p<0.0001) and wet body mass (p=0.005), and explained about 38% of the total variability (R^2 =37.7%; adjusted R^2 =35.2%; Fig. 2.5). Based on the results of ANOVA, the dose of 2400 mg Ni kg^{-1} food (the lowest observed effect concentration, LOEC) was chosen to study effects of interaction between temperature and Ni exposure on beetles respiration rates in the second part of the study (Table 2.2).

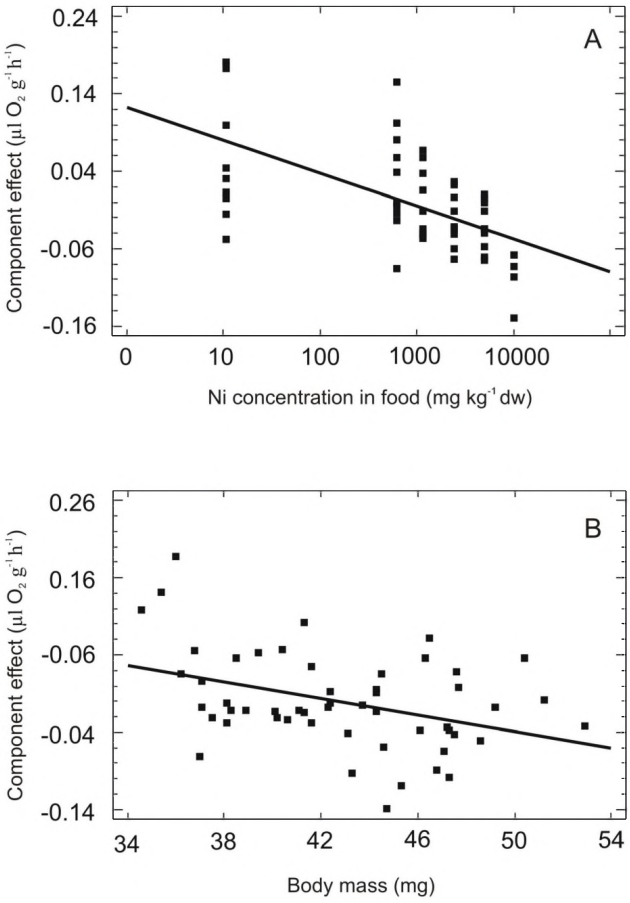

Fig. 2.5. Results of the multiple regression analysis: effect of Ni concentration in food (A) and body mass (B) on respiration rate of *Pterostichus oblongopunctatus* in the Experiment I. The model is significant at $p<0.0001$; $R^2=0.38$. The line shows the relative change in the predicted values of respiration rates that occurs when changing Ni concentration (A) and body mass (B) over their observed ranges. Each point is then plotted by adding its residuals to a line. The whole model is significant at $p<0.0001$, and the respiration rate correlates with Ni concentration at $p<0.0001$ (A), and with body mass at $p=0.005$.

Experiment II

The statistical analysis indicated significant effects of the following variables on the respiration rates: temperature (p<0.0001; Fig. 2.6), day of exposure (p=0.006), series (p=0.04) and wet body mass (p=0.003) with no significant interactions between the variables (Table 2.3 and 2.4). Thus, in contrast to the Experiment I, no significant overall effect of Ni on the respiration rates was observed (p=0.17). When the effects were analyzed separately for each day, it appeared that at 16th d of the experiment the respiration rates of beetles originating from 10°C were significantly higher than of those from 15°C and 20°C (p=0.007), and the only significant variable in the model was the temperature. At 32nd d of the contamination period the respiration rates did not differ between animals originating from 10°C and 15°C, and both were significantly higher than in the beetles originating from 20°C (p=0.001). Similar relationship was found at the end of the contamination period (day 64; p=0.04). Apart from the temperature, the series and wet body mass were significant in the model at 32nd d (p=0.01 and p=0.02, respectively) and the wet body mass (p=0.004) at 64th d of the experiment. After the depuration period (day 128) no effect of any variable on the respiration rate was found.

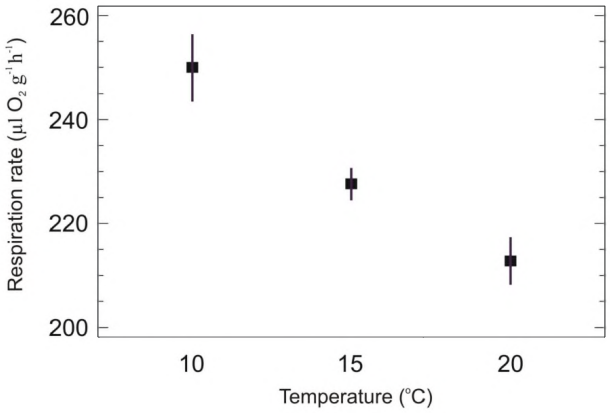

Fig. 2.6. Respiration rates of *Pterostichus oblongopunctatus* at different temperatures in the Experiment II (mean ± SE); overall temperature effect significant at p<0.0001, all temperatures significantly different from each other at p<0.05. Other significant factors were: day of exposure (p=0.006), series (p=0.04), and body mass as a covariate (p=0.003).

Table 2.3. Respiration rates of *Pterostichus oblongopunctatus* measured for each treatment at 0, 16, 32, 64 and 128 days after the start of the Experiment II (mean ± SD).

Day	Temperature											
	10				15				20			
	Control		Ni-treated		Control		Ni-treated		Control		Ni-treated	
	Series I	Series II	Series I	Series II	Series I	Series II	Series I	Series II	Series I	Series II	Series I	Series II
0	238 ± 46 (9)*	–	–	–	216 ± 20 (10)	–	–	–	214 ± 39 (10)	–	–	–
16	301 ± 70 (5)	–	327 ± 100 (4)	–	212 ± 17 (3)	–	235 ± 26 (4)	–	232 ± 27 (5)	–	261 ± 3 (2)	–
32	270 ± 59 (4)	223 ± 38 (5)	297 ± 89 (3)	232 ± 17 (4)	296 ± 17 (2)	215 ± 13 (5)	222 ± 29 (5)	220 ± 20 (5)	214 ± 57 (4)	203 ± 30 (5)	195 ± 35 (5)	180 ± 11 (3)
64	211 ± 35 (5)	234 ± 27 (5)	227 ± 32 (5)	223 ± 37 (4)	234 ± 28 (5)	228 ± 44 (5)	241 ± 43 (5)	218 ± 1.0 (3)	231 ± 48 (4)	219 ± 28 (5)	192 ± 36 (6)	172 ± 46 (4)
128	249±43 (5)	239±26 (6)	249±78 (5)	274±14 (4)	237±14 (5)	232±12 (4)	231±9.5 (3)	223±14 (6)	251±20 (6)	214±26 (5)	201±53 (4)	219±8.0 (3)

* number of beetles used for mean and SD calculation; beetles which died in the course of the respiration rate measurements and 8 outliers were excluded from the analysis.

When the data were analyzed separately for each culturing temperature, significant decrease of respiration rates was found in animals exposed to Ni at 20°C (p=0.04). Also, effects of the series and wet body mass were significant (p=0.04 and p=0.004, respectively).

Table 2.4. Summary of multifactor analysis of variance for rank respiration rates of *Pterostichus oblongopunctatus* in the Experiment II*.

Model term	Sum of squares	*df*	Mean square	*F*-ratio	*p*-value
COVARIATES					
Body mass	30492.0	1	30492.0	9.17	0.003
MAIN EFFECTS					
Temperature	89881.6	2	44940.8	13.51	<0.0001
Day	48941.1	4	12235.3	3.68	0.006
Series	13668.8	1	13668.8	4.11	0.04
RESIDUAL	681685.0	205	3325.29		
TOTAL (CORRECTED)	858347.0	213			

* Only significant variables are included, *df* = degrees of freedom

Discussion

The data obtained in the present study revealed toxic effect of Ni on *P. oblongopunctatus*. This was expressed as decreased survival and respiration rates of Ni exposed beetles. It was also found that the temperature at which the beetles were cultured affected their respiration, even if the respiration rate was always measured at the same constant temperature for all beetles (20°C).

Nickel, like many other metals (e.g. Cu, Zn, Fe), is an essential element for many species and either deficiency or toxicity symptoms may occur when too little or too much Ni is assimilated. In the present study, lower levels of dietary Ni increased survival in the beetles by about 43%, relative to the control with no Ni added. This effect was statistically significant (Fig. 2.3) and suggests that the beetles may require

Ni at somewhat elevated levels. Nevertheless, at high concentrations Ni was clearly toxic as indicated by the LC_{50} value (8351 mg Ni kg^{-1} for 245 d of exposure). The high concentrations of Ni can cause alterations in distribution and transport of other elements, such as zinc and copper (Przybyłowicz et al., 2002), which may magnify the toxic effect of Ni.

Nickel concentration in the beetles increased steadily with increasing concentration in food (Fig. 2.4). However, beetles survival was affected by Ni only at the highest level tested. Thus, at the range of environmentally realistic concentrations, Ni does not seem highly toxic, although after long exposure to extremely high pollution levels increased mortality may occur. Also, no significant effect of Ni on reproduction was found, but this might be due to the low number of replicates (three to five pairs per treatment) and high variability in the numbers of eggs produced by this species (Łagisz et al., 2002). Earthworms and springtails seem more sensitive than the ground beetles to this metal (Scott-Fordsmand et al., 1998, Scott-Fordsmand et al., 1999; Lock and Janssen, 2002). It has to be noticed however that Ni content in animals vary vastly among species. For example, earthworms from uncontaminated soils may contain as much as 38 mg Ni kg^{-1} dw (Eisler, 1998) whereas the concentration of Ni in control beetles from the Experiment I was about 15 times lower (Table 2.1). The highest Ni concentrations recorded in invertebrates reach 950 mg kg^{-1} (in grasshoppers of the *Stenoscepa* genus; Boyd et al., 2007) or even 5000 mg kg^{-1} (in workers of two termite species, *Odontotermes transvaalensis* and *Trinervitermes dispar*; Eisler, 1998), while at the highest treatment in the Experiment I a concentration of 725 mg kg^{-1} was reached in the ground beetles, and at the second highest treatment it was only 127 mg kg^{-1}. Thus, the rather low toxicity of Ni observed in the present study might result from high elimination efficiency for this metal in carabids.

Detoxification of metals is connected with energetically costly processes (e.g., production of metallothioneins and metal-containing granules; Hopkin, 1989, Walker et al., 2006) and should cause an increase in metabolic rate (Sibly and Calow, 1989). On the other hand, the opposite response to intoxication, that is a decrease of the respiration rate, is also possible due to damage of respiratory enzymes (Laskowski et

al., 1996). Indeed, elevated respiration rates were observed in ground beetles (*P. oblongopunctatus*) collected from metal polluted sites (Lagisz et al., 2005) and in laboratory strains of confused flour beetle (*Tribolium confusum*) treated for several generations with copper (Lukasik and Laskowski, 2007). Rowe et al. (2001) also showed increase of daily standard metabolic rate in the crayfish *Procambarus acutus* exposed to sediments and food contaminated with trace-element, but this effect was not retained during longer-term exposure. Similarly, the respiration rate of centipedes treated with copper was elevated for a short period only, and later returned to the same level as in control animals (Laskowski et al., 1996). The opposite reaction – a decrease in the respiratory metabolism, was found in house crickets (*Acheta domesticus*) intoxicated with cadmium, while zinc or lead did not affect their respiration rate (Migula, 1989). Thus, effect of metals on respiration rate seems to depend on the species, the metal used and its concentration as well as the exposure time. Despite the growing interest in fate of Ni in the environment, relatively little is known about its toxicity and effects on respiratory metabolism of invertebrates. In the present study, clear negative effect of Ni on the beetles respiration rate was observed in Experiment I. This indicates that after approximately half a year of exposure (192 d) the beetles suffered probably from damage of some enzymes, and the damage was dose-dependent as suggested by decreasing respiration rates with the increase in Ni concentration in food.

The dose of 2400 mg Ni kg^{-1} food was the lowest that after 192 d of exposure significantly reduced respiration rates in comparison to control (Table 2.2). However, when animals were exposed to such a dose in the second, shorter experiment, the metabolic rates of Ni-treated beetles either did not differ from control animals throughout the experiment or the effect was minor and statistically significant only in the beetles living at 20°C. Most probably, the exposure time (64 d) in the Experiment II appeared too short to exert negative effects on the beetles' metabolism. In comparison to the life cycle of *P. oblongopunctatus* (about one year), 64 d cover about 1/6 of its life span. It is pretty much in accord with other tests used in terrestrial ecotoxicology, but still may miss some important effects which occur after longer

exposure. For example, Laskowski (2001) concluded from his study on cadmium and pesticide effects on aphids that whenever the accumulation of a chemical throughout the life span of an individual is expected, ecotoxicological experiments should cover at least 2/3 of the total life span.

No interaction between Ni treatment and temperature was found. Only when the data were analyzed separately for each temperature, the differences between Ni treatments were found; the Ni-treated animals originating from 20°C had lower respiration rates than control, indicating a trend similar to that found in the Experiment I. The weak or non-existent effects of Ni in the Experiment II suggests that only after long-term exposure to Ni, which is generally rather poorly accumulating metal (Kabata-Pendias, 2000), the animals suffered from its direct toxicity. However, this should be treated as a presumption only, as apart from time of exposure, the two experiments differed also in the origin of the beetles (laboratory-cultured *vs* field-collected animals) and their age, which also could influence their respiratory metabolism and sensitivity to Ni. Additionally, in the second experiment only males were used for respiration rate measurements: firstly, because during the first weeks of beetles activity in April males dominate, thus it was possible to catch only enough males for the whole experiment, and secondly, because *P. oblongopunctatus* mate since April/May (Brunsting, 1981), so it was highly probable that at least some of the caught females could be already fertilized, and this is known to influence metabolism. To test the hypothesis that negative effects of Ni intoxication appear only after relatively long exposure, resulting in substantial accumulation of Ni in beetle bodies, would require a separate study in which the levels of Ni accumulated would be measured immediately after respiration measurements. Unfortunately, this could not be done in this study: in the Experiment I beetles were used for reproduction measurements and, thus, could not be killed immediately after the respiration measurement. The level of Ni was measured 7 weeks after the respiration rates were measured. In turn, in the Experiment II the same individuals were used for respiration measurements at different days.

The pattern of the relationship between the respiration rate and temperature at which the animals were cultured changed over time, but during whole contamination period

the animals originating from 20°C had always the lowest respiratory metabolism (Fig. 2.6). Possibly, the more active animals at 20°C accumulated Ni faster than at lower temperatures. Alternatively, the increased respiration rate in animals originating from lower temperatures could be related to increased activity of the beetles when transferred to 20°C for respiration rate measurements.

Assuming that respiratory metabolism is an indirect measure of organism's maintenance costs, the lack of statistically significant effect of Ni on respiration rate in the Experiment II may suggest that during the exposure time of 64 d the animals did not incur energetic costs connected with toxicant elimination or toxicant-induced cellular damage reparation (Calow, 1991). To confirm this, more studies linking the respiration rate to metal kinetics need to be done. Also the future biochemical studies of detoxification enzymes in Ni-treated beetles seem promising for their potential use as biomarkers of physiological stress. Although the physiological data, such as respiration rates and/or enzyme activity are complex and sometimes difficult to interpret, their intimate link with organism performance is worth investigating, especially when related to endpoints often used in ecological risk assessment such as survival and reproduction.

Acknowledgements

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CHAPTER 3

COMBINED EFFECT OF ENVIRONMENTAL POLLUTANTS (NICKEL, CHLORPYRIFOS) AND TEMPERATURE ON THE GROUND BEETLE, *PTEROSTICHUS OBLONGOPUNCTATUS*

This chapter has been published as:

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Abstract

Terrestrial organisms in the field are often exposed to a combination of stress factors of various origin, but little is known about interactions between different types of stressors. In the present study, the results of a study on interactions between nickel (Ni), chlorpyrifos (CPF) and temperature (T) in the ground beetle, *Pterostichus oblongopunctatus* are demonstrated. The results revealed that all factors, and their interactions, influenced life-cycle parameters of the beetles (survival and reproduction). Significant three-factor interactions were found for effects on beetle survival, indicating that the combined negative effect of Ni and CPF was temperature dependent. In addition, significant effects of body mass were found: the survival of beetles treated with CPF, and the reproduction of beetles exposed to Ni were positively correlated with body mass. All studied endpoints were affected by temperature. The results indicate that understanding interactions between temperature and toxicants, as well as among chemicals themselves, is essential for proper ecological risk assessment.

Keywords: multiple stress, metals, pesticides, temperature, ecological risk assessment

Introduction

In the real world, organisms constantly respond to environmental conditions, and it is obvious that some factors can be far from optimal for many organisms and can be perceived by them as "stressors". These "stressors" can range from such natural factors as drought, radiation or unfavorable temperatures to naturally occurring or anthropogenic toxic chemicals. All these factors may interact with each other, and the simultaneous exposure to fluctuating physical and chemical parameters may cause organisms to respond in a way that is not possible to predict from single-factor studies.

Decades of detailed studies regarding the toxicity of various chemicals and their mixtures have provided reasonably abundant data for dozens of organic and inorganic pollutants and paved the way for several approaches toward risk assessment of toxicant combinations. Certainly, however, many natural physical and chemical parameters of the environment interact with chemicals either directly (e.g., changing their bioavailability) or indirectly (by changing organisms biology and/or behavior) (Sjursen and Holmstrup, 2004) Knowledge about these interactions and how they affect organisms' fitness is surprisingly scarce (Parker et al., 1999). Probably the most important natural factor, which may be highly variable in the field and is of major importance for the physiological state of an organism, is temperature. Those few ecotoxicological studies in which effects of temperature were investigated in terrestrial invertebrate species (Sjursen and Holmstrup, 2004; Abdel-Lateif et al., 1998; Holmstrup et al., 2000) confirm its importance for toxic effects of pollutants and may lead to better extrapolation of results from standard ecotoxicological assays to field conditions.

The present study describes the effects of multiple interactions between toxicants and a non-chemical factor in the ground beetle, *Pterostichus oblongopunctatus* (Coleoptera: Carabidae). Two chemicals with different modes of action and different behavior in the environment were used in the present study: a pesticide (chlorpyrifos) and a metal (nickel). In many metal-polluted areas, animals may be exposed simultaneously to pesticide sprays in agricultural and horticultural systems. Pesticides

are highly toxic but gradually degrade after application. In contrast, toxicity of metals is relatively low, but they do not degrade and have a tendency to accumulate in the environment and organisms (Laskowski, 2001). Carabids represent an important level in the food chain and are found to be poor accumulators of metals (Kramarz, 1999a), which may results from efficient mechanisms of detoxification and excretion, but Stone et al. (2001) showed that beetles inhabiting metal-polluted environments are less tolerant to additional environmental stressors (food deprivation or a pesticide) than those from less-contaminated areas. Therefore, even if metals are not highly toxic themselves, they may have detrimental impact on beetles when exposure occurs in combination with other chemicals and/or non-chemical stressors.

To make the study as realistic as possible, ground beetles were exposed chronically to a metal (Ni) but only to a single pesticide (CPF) application. Industrial uses of Ni have resulted in the redistribution of a significant amount of this metal into the environment. Near smelters, Ni concentrations in soils may exceed 6500 mg kg^{-1} (Stefanowicz et al., 2008), and a concentration as high as 22,000 mg Ni kg^{-1} was found in smelter-contaminated soil by Everhart et al. (2006). Some insects such as South African beetle *Chrysolina pardalina*, feeding on a Ni-hyperaccumulating plant species, *Berkheya coddii*, have adapted to the Ni-rich environment (Augustyniak et al., 2007). However, Ni can be toxic for soil invertebrates, such as earthworms (Scott-Fordsmand et al., 1998; Lock and Janssen, 2002) and springtails (Scott-Fordsmand et al., 1999). Earlier study (Chapter 2) demonstrated that after long exposure to Ni in *P. oblongopunctatus*, some negative effects, such as reduced respiration rates and increased mortality, appear. As a pesticide, an acetylcholinesterase (AChE) inhibitor, CPF, was chosen for the present study. Chlorpyrifos is widely used in agricultural crops, rangelands, forests and wetlands because of its effectiveness and toxicity to target organisms (Cox, 1995). Like many organophosphorus pesticides (OPs), however, it is also toxic to non-target organisms, especially insects. Many beneficial insects, such as parasites, parasitoids, pollinators and predators (including carabid beetles), may be affected by CPF (Cox, 1995). According to label recommendation for winter wheat, CPF is applied at a dose of 480 g active ingredient (a.i.) ha^{-1}. Assuming that this dose is applied in a volume of

1000 L, it results in a concentration of 480 ng μl^{-1}. The small droplets of 1 µl or 0.5 µl are the common size in the field pesticide application; however, the large range of droplet sizes may be produced by different application equipment. As a non-chemical factor, temperature (T) was used. Temperature influences physiological processes of beetles, such as by altering their respiratory rate, feeding activity, and locomotor activity, and thus can modify the organisms exposure and response to chemicals. Once effects of single factors (Ni, CPF, T) have been established in preliminary studies, the effects of their combination were investigated in a full factorial design with three levels of each factor (3×3×3). It was especially interesting to examine the extent to which joint effects of multiple interactions affected survival and reproduction of adult beetles.

Materials and Methods

Pterostichus oblongopunctatus culture

The ground beetles for the main part of the experiment were taken from a laboratory culture maintained at the Institute of Environmental Sciences; this culture was established from approximately 120 adult beetles collected at an uncontaminated site near Krakow, southern Poland. More detailed information about the culture conditions has been given in Chapter 2. For a range-finder study adult ground beetles were collected in the field, in the same area as the beetles used to establish the laboratory culture.

Experimental design

First, range-finding experiments were performed with single stressors to determine the susceptibility of *P. oblongopunctatus* to Ni, CPF and temperature. The aim of the range-finding was to select such ranges of Ni concentrations in food and CPF doses that would result in survival times long enough to allow the effects of the treatments on both the death rate and the reproductive output to be differentiated. Based on the results, nominal Ni concentrations of 0 (control), 5000 and 10,000 mg kg^{-1} dry food (Ni-0, Ni-5000 and Ni-10000, respectively) and CPF doses of 0, 40 and 80 ng a.i. $beetle^{-1}$ (CPF-0, CPF-40 and CPF-80, respectively) were used for further study. Nickel

chloride hexahydrate ($NiCl_2 \times 6H_2O$; Eurochem BGD, Warsaw, Poland) was added to the food as aqueous solution, and CPF (minimum purity, 98%; technical grade; Cheminova, Lemvig, Denmark) was diluted in acetone and dosed topically. The preliminary experiments showed that at temperatures below 10°C and above 30°C, the beetles basically did not reproduce; thus, the temperatures of 10, 20 and 25°C (T-10, T-20 and T-25, respectively) were chosen for further study. At the two extreme temperatures used, 10°C and 25°C, reproduction could be affected directly, and because of the strong dependency of invertebrate metabolism on temperature, detoxification processes could be altered as well. These temperatures are within the range of temperatures observed in the field during the reproductive season.

The full factorial design was used with three levels of each factor (Ni, CPF, and T). Altogether, 232 beetles (113 females and 119 males) were used in the experiment. The experiment started when the laboratory-cultured beetles were transferred to the second-week-of-spring conditions: 12:12 h light:dark photoperiod (L:D), 14:12°C L:D, relative humidity (RH) at 75%, and light intensity of 300 lx (see Chapter 2). At this point, the animals were randomly allocated to three experimental groups fed either Ni-contaminated (5000 or 10,000 mg kg^{-1}) or identical, uncontaminated food. After one week, the animals were transferred to the third- and then to the fourth-week-of-spring conditions (12:12 h L:D, 16:14°C L:D, 75% RH, 300 lx and 14:10 h L:D, 18:16°C L:D, 75% RH, 300 lx, respectively) and then were kept at summer conditions (16:8 h L:D, 20°C, 75% RH, 500 lx) for the next week. A number of earlier toxicokinetic studies suggest that a four-week period is long enough to reach an equilibrium body metal level (if such a level does exist for a particular metal) (Kramarz, 1999a; Janssen, 1991). After this period, the beetles were weighed to the nearest 0.001 g (WPA 180/k Radwag, Radom, Poland) and then dosed topically either with 1 µl of pure acetone (control) or with 40 or 80 ng a.i. of CPF dosed in 1 µl of acetone. One-microliter drops were applied along the suture line between the elytra and the pronotum using Hamilton gas-tight syringe with a semiautomatic dispenser. The beetles were then split into three groups, which were transferred to climatic chambers differing only in ambient temperature (10, 20 or 25°C). Following the dosing, the beetles were observed

constantly for 2 h. Survival was checked during the Ni pretreatment period every third day and after the CPF dosing at approximately 3, 6, 12, 24, 36 and 48 h; then once a day for the next 30 d; and every third day thereafter. For the first 48 h, the beetles were kept individually in plastic vials. Afterward, those that survived were coupled according to the treatment, and each pair of beetles was kept in a separate, 100-ml plastic box. Four pairs were used for each treatment. The boxes were filled to approximately three-fourths with moistened peat (pH_{H_2O} 4.5-5.0, 80% water holding capacity, WHC). Every third day, the eggs laid by the beetles were picked out and deposited individually on moist filter paper in 24-well tissue culture plates. The plates were stored at 20°C in darkness and controlled daily for newly hatched larvae. The larvae and emerged imagines were fed *ad libitum* with uncontaminated artificial food (the same as in the laboratory culture; i.e., ground mealworms mixed with ground apple with 1 g sodium benzoesate ($C_7H_5NaO_2$; Fluka, Deisenhofen, Germany) per 1 kg food as preservative) every third day.

The recorded endpoints were the lifetime (followed for 134 d since the pesticide application) and the reproduction rate, expressed as number of eggs, larvae, and adults produced per female.

Chemical analyses

To determine actual Ni concentrations in food, approximately 0.1 g dry sample of each batch was digested in 2 ml concentrated HNO_3 (Merck, Darmstadt, Germany) with a gradual increase of temperature from 50 to 150°C. When a sample was mineralized, the excess of HNO_3 was evaporated to the volume below approximately 0.05 ml, and the sample was diluted to 10 ml with deionised water. Blank samples and samples of certified reference material (dogfish muscle DORM-2; National Research Council, Ottawa, ON, Canada) were included to check analytical precision. Concentrations of Ni were measured by flame atomic absorption spectrometry (AAnalyst 800; Perkin-Elmer, Boston, MA, USA). The detection limit (0.68 mg L^{-1}) was calculated as three standard deviations for the mean of the calibration blank measurements. The measured

concentrations were within ± 13% of the certified reference value. Nickel concentration was expressed in mg kg^{-1} dry weight.

Statistical analyses

Data for all parameters were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). When those conditions were not met, statistical analyses were performed on ranks. Rank transformation was chosen because the variance of rank data is automatically stable.

Differences in the body mass of beetles between treatments and sexes were analyzed with two-way analysis of variance (ANOVA) on ranks. Although females differed significantly from males in body mass, both sexes were combined for survival analyses as a function of time because of the low number of individuals. If a test conducted on all treatments detected statistically significant difference among survival curves ($p \leq 0.05$), they were analyzed separately within each factor (Ni, CPF or T) by conducting pair-wise comparisons using log-rank test (Mantel, 1966). For treatments in which mortality during the experiment was large enough, the median survival time LT_{50} was estimated from the survival analysis (Kaplan and Meier, 1958).

To quantify the relationships between the endpoints (lifetime and reproduction) and the factors (Ni, CPF and T), the data were analyzed with the general linear models (GLM) method. Lifetime was expressed in days, and ranked number of eggs was used as the fecundity measure. The analysis started with formulating the full model – that is, testing all main factors and interactions for significance ($p \leq 0.05$). For temperature, a quadratic term was included in the initial model, because the relationships between temperature and many traits of organisms are best described by a quadratic model (a parabola), with an optimal temperature and lower and upper pessima. Because of the limited number of degrees of freedom, however, temperature was used only as a liner term in the interaction terms. Additionally, significance of the beetle body mass was tested in the present experiment, because all beetles were dosed with the same amount of CPF. Therefore, its effect on survival could be mass dependent. Also, the reproductive output frequently is related to body mass. The initial full model was thus:

$$Y = a1 + a2{\times}T + a3{\times}Ni + a4{\times}CPF + a5{\times}mass + a6{\times}T^2 + a7{\times}T{\times}Ni + a8{\times}T{\times}CPF + a9{\times}T{\times}mass + a10{\times}Ni{\times}CPF + a11{\times}Ni{\times}mass + a12{\times}CPF{\times}mass + a13{\times}T{\times}Ni{\times}CPF + a14{\times}T{\times}Ni{\times}mass + a15{\times}T{\times}CPF{\times}mass + a16{\times}Ni{\times}CPF{\times}mass + a17{\times}T{\times}Ni{\times}CPF{\times}mass$$

where Y is the dependent variable studied, and $a1$ to $a17$ are the estimated parameters. After running the initial model, the nonsignificant terms were consecutively removed from the model, starting with those with the highest p value, as long as only factors significant at $p \leq 0.05$ remained.

All beetles used in the experiment were taken into account during survival and GLM analysis. The lifetime of beetles that survived until the end of the experiments was set as equal to the test duration (134 d). This approach may have led 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 were smaller than if the duration of the experiment would have been prolonged. Those females that did not survive the first 48 h after CPF application, and thus, were not paired, as well as those that were paired but did not reproduce, were assigned zero reproductive rate, because in both cases, the consequences for the population growth rate were the same.

All analyses were done using Statgraphics Centurion XV (StatPoint.; http://www.statgraphics.com).

Results

The measured Ni concentrations in food were in good accordance with assumed nominal values. The average concentration (mean ± standard deviation) of Ni in control was 10.6 ± 4.2 mg kg^{-1} dry weight and 5174 ± 271 and 9495 ± 294 mg kg^{-1} at nominal concentrations of 5000 and 10,000 mg kg^{-1}, respectively.

The mean body mass measured after the four-week Ni exposure was 0.0469 g for females and 0.0417 g for males, and the difference was significant at $p<0.0001$. No statistically significant differences in body mass were found between beetles assigned to different treatments.

Survival

Because all beetles survived the four-week Ni pretreatment period irrespective of the Ni concentration in food, survival was assessed for 134 d after the application of CPF. Chlorpyrifos caused high mortality at the beginning of the experiment (especially in males), but some animals had survived equal to control beetles. The first male died after 17 h (0.71 d) at Ni-5000/CPF-40/T-20, and the first two females died after 40 h (1.65 d), at Ni-5000/CPF-80/T-25. Survival curves for all exposure conditions (27 treatments) differed significantly ($p<0.0001$) and were analyzed consecutively within each Ni, CPF, or temperature group. Survival of beetles exposed to various Ni concentrations at each temperature and pesticide combination (3×3) is shown on Fig. 3.1. All control beetles at all temperatures survived until the end of the experiment. At 10,000 mg kg^{-1}, Ni decreased the survival in all treatments, and the highest mortality was seen among animals dosed with 80 ng CPF $beetle^{-1}$ at two marginal temperatures, 10 and 25°C : LT_{50}s (mean ± standard error) were 2.54 ± 1.22 d and 2.65 ± 0.68 d at 10 and 25°C, respectively, compared to 15.7 ± 20.2 d at 20°C. Because of the high mortality caused by CPF in these treatments, however, no differences in survival curves between Ni concentrations were found. Only at 20°C was the mortality of beetles dosed with 80 ng CPF $beetle^{-1}$ and fed with either 5000 or 10,000 mg Ni kg^{-1} significantly higher than that of beetles fed uncontaminated food ($p\leq0.002$). Moreover, no differences between 0 and 5000 mg Ni kg^{-1} at 10°C as well as between 5000 and 10,000 mg Ni kg^{-1} at 25°C, were found in the beetles exposed to 40 ng CPF $beetle^{-1}$, whereas their lifetime was significantly lower at 10,000 mg Ni kg^{-1} in comparison with those fed uncontaminated food at all temperatures (p values at least 0.0006) when either 0 or 40 ng CPF $beetle^{-1}$ were used (Fig. 3.1).

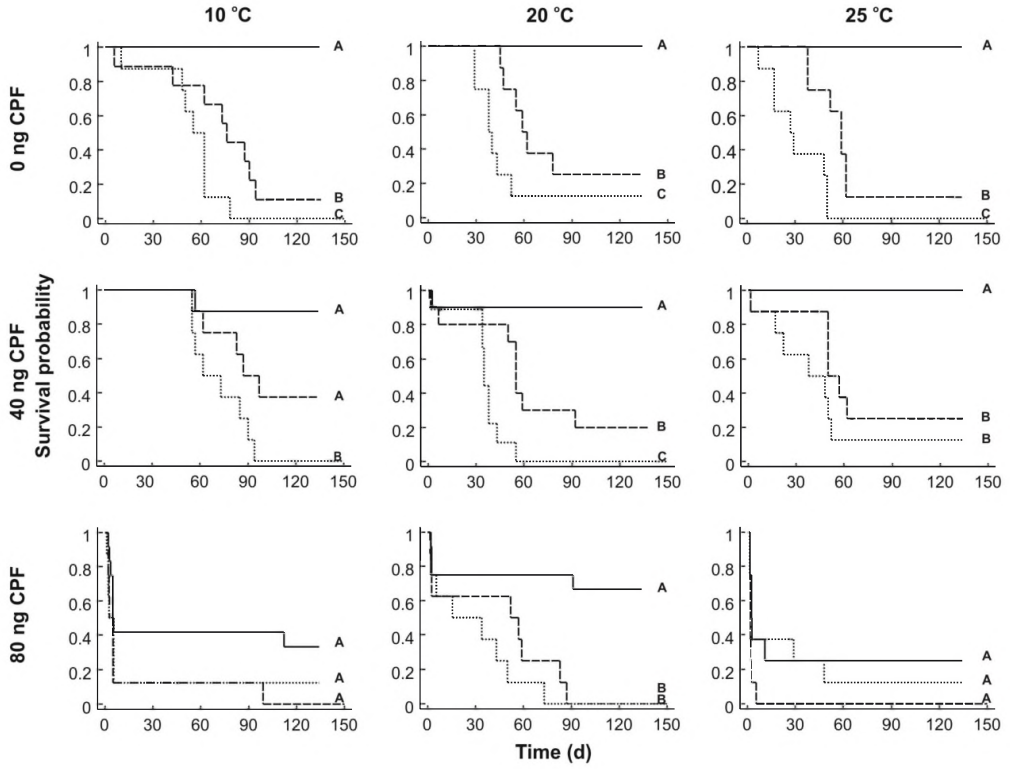

Fig. 3.1. Survival of *Pterostichus oblongopunctatus* exposed to various Ni concentrations in food (— 0, - - 5000 and ··· 10,000 mg Ni kg^{-1}) at 10, 20 and 25°C and 0, 40 and 80 ng chlorpyrifos (CPF) $beetle^{-1}$ as a function of time. The same letter means no significant differences in lifetime between the Ni treatments.

Chlorpyrifos significantly increased mortality in the beetles not treated with Ni at 10 and 25°C, but no differences between temperatures were found at the two doses of CPF used. Additionally, no differences between temperatures were found in the beetles exposed to either 5000 mg Ni kg^{-1} or to a combination of 5000 mg Ni kg^{-1} and 40 ng CPF $beetle^{-1}$. Only those exposed simultaneously to 5000 mg Ni kg^{-1} and 80 ng CPF $beetle^{-1}$ survived significantly better at optimal temperature compared with 25°C. The effect of temperature also was seen in beetles fed with 10,000 mg Ni kg^{-1}, in which, depending on the pesticide dose, the differences between 10 and 25°C (no CPF) or between 10 and 20°C (40 ng CPF $beetle^{-1}$) were observed. In general, a strong effect of the highest Ni concentration and the highest CPF dose was why no effect of

temperature was found when these two toxicants were used in combination at their highest rates.

The statistical analysis indicated significant effects of the following variables and/or their interactions on the lifetime: Ni, CPF, T^2, CPF×mass and Ni×CPF×T (Table 3.1). The final model (i.e., the one with significant variables and interactions only) was:

$$lifetime = 146.5 - 0.0085 \times Ni - 2.34 \times CPF - 0.0345 \times T^2 + 34.96 \times mass \times CPF + 0.00000241 \times Ni \times CPF \times T$$

The final model was significant at $p<0.0001$ and explained approximately 47% of the total variability in the life span of *P. oblongopunctatus* (R^2 =47.4%; adjusted R^2 =46.2%; Fig. 3.2A).

Table 3.1. Type III sums of squares and significance levels of the variables for the final general linear models for lifetime of *Pterostichus oblongopunctatus* treated with Ni and chlorpyrifos (CPF) at different temperatures (T)*.

Model term	Sum of squares	*df*	Mean square	*F*-ratio	*p*-value
Ni	133208.0	1	133208.0	89.55	<0.0001
CPF	61424.5	1	61424.5	41.24	<0.0001
T^2	10428.5	1	10428.5	7.01	0.009
mass×CPF	29001.3	1	29001.3	19.50	<0.0001
Ni×CPF×T	11557.2	1	11557.2	7.77	0.006
RESIDUAL	336186	226	1487.6		
TOTAL (CORRECTED)	638775	231			

* Only significant variables are included, *df* = degrees of freedom

Although direct effects of both Ni and CPF were negative (*p* values less than 0.0001 for both), the heavier animals were able to survive longer when treated with the pesticide ($p<0.0001$). A weak but clearly significant ($p=0.006$), second-order interaction was found between the two pollutants and temperature, indicating not only that the toxicity of the chemicals was affected by each other but also that this

interaction depends on temperature. In general, the animals treated with, for example, Ni survived longer if they were exposed to CPF at lower temperatures. This can be partly seen from Fig. 3.3, which shows lifetime isoclines for beetles treated with different combinations of Ni and CPF at two extreme temperatures: 10 and 25°C. The survival was also parabolically related to temperature (p=0.009).

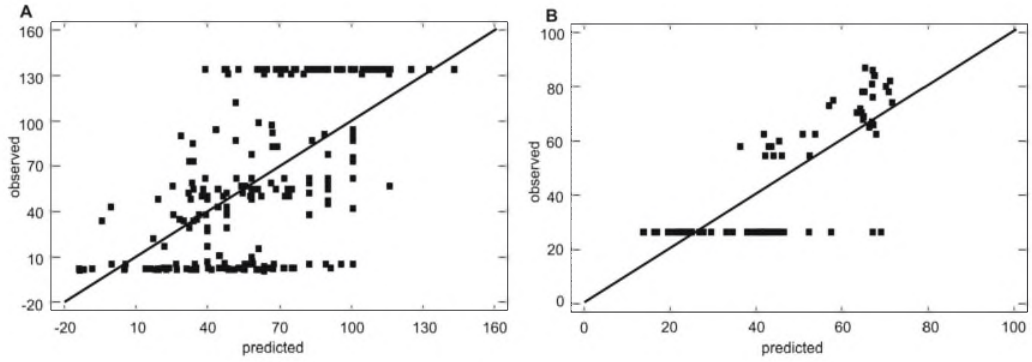

Fig. 3.2. Observed values of lifetime (A) and ranked number of eggs (B) versus values predicted by the fitted model. The closer the points lie to the diagonal line, the better the model at predicting the observed data.

Fecundity

For this part of the study, the number of replicates (i.e., pairs that were used in the reproduction) depended on survival after CPF dosing. This was a problem especially at the highest CPF dose, at which only few individuals survived until coupling. In all 80 ng CPF treatments, the number of replicates was lower than four, and in three treatments (Ni-10000/CPF-80/T-10, Ni-10000/CPF-80/T-25 and Ni-5000/CPF-80/T-25), not a single pair entered the reproduction. No female reproduced at the highest Ni concentration, and at least two non-reproducing females were found at 5000 mg kg^{-1} irrespectively of CPF dose and temperature. The beetles laid 516 eggs altogether. All control beetles together laid 69, 128 and 4 eggs at temperatures 10, 20 and 25°C, respectively. The proportion of hatched larvae from eggs laid by control beetles at 10 and 20°C was 20% and 40%, respectively. Because of the low number of hatched

larvae and emerged imagines, only the number of eggs produced per female was used for statistical analyses of effects on fecundity.

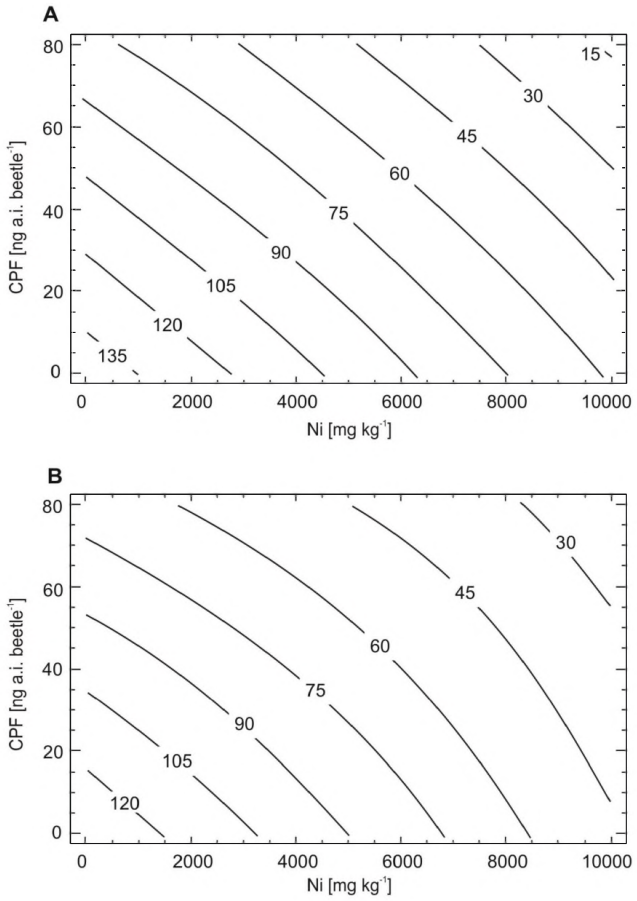

Fig. 3.3. Effect of Ni and chlorpyrifos (CPF) on lifetime (number of days marked on isoboles) of *Pterostichus oblongopuncatus* at different temperatures (T): isobolic representation of the interaction (p=0.006) between Ni and CPF at 10°C (A) and 25°C (B). Note that the interaction depends on temperature, as indicated by a significant, second-order order interaction Ni×CPF×T. Both graphs plotted at average body mass (0.044 g). a.i. = active ingredient.

The GLM describing the relationship between egg production (as ranks) and temperature, body mass, Ni concentration in food, and CPF dose indicated that the following variables and/or their interactions were significant: T^2, mass×Ni, mass×T and Ni×T (Table 3.2).

The final model was:

$$rank(eggs) = 70.48 - 0.109 \times T^2 - 0.148 \times mass \times Ni + 53.99 \times mass \times T + 0.000156 \times Ni \times T$$

The model was significant at $p<0.0001$ and explained approximately 47% of the total variability (R^2 =46.8%; adjusted R^2 =44.8%; Fig. 3.2B).

Table 3.2. Type III sums of squares and significance levels of the variables for the final general linear models for rank fertility (number of eggs per female) of *Pterostichus oblongopunctatus* treated with Ni and chlorpyrifos (CPF) at different temperatures (T) *.

Model term	Sum of squares	*df*	Mean square	*F*-ratio	*p*-value
T^2	4675.9	1	4675.9	11.76	0.0009
mass×Ni	10590.0	1	10590.0	26.64	<0.0001
mass×T	3514.0	1	3514.1	8.84	0.004
Ni×T	1928.3	1	1928.3	4.85	0.03
RESIDUAL	42936.3	108	397.6		
TOTAL (CORRECTED)	80676.0	112			

* Only significant variables are included, *df* = degrees of freedom

Significant interactions were found between Ni and temperature (p=0.03; Fig. 3.4), between body mass and temperature (p=0.004), and between body mass and Ni ($p<0.0001$, Fig. 3.5). Similar to the lifetime, the quadratic relationship of fecundity on temperature also was visible.

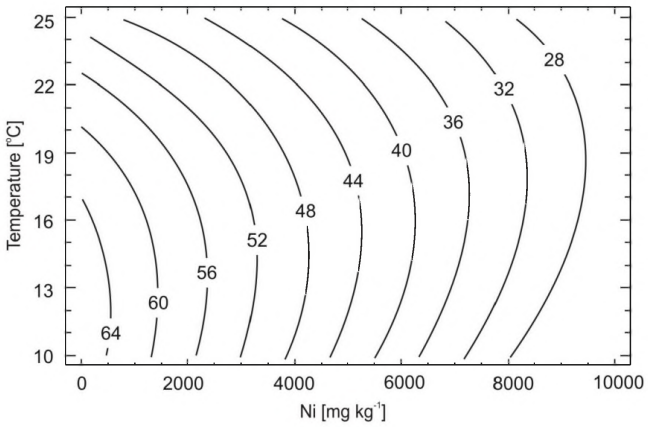

Fig. 3.4. Temperature-dependent Ni effect on fecundity (ranked number of eggs marked on isoboles) of *Pterostichus oblongopuncatus*: isobolic representation of the interaction (p=0.03) between Ni and temperature shown for beetles not exposed to chlorpyrifos (CPF) at average body mass (0.044 g). Note the quadratic relationship of *P. oblongopuncatus* fecundity on temperature.

Discussion

Separate effects of metals, pesticides and temperature on invertebrates are well documented. Some information about joint effects of chemical and non-chemical factors also are available for terrestrial invertebrates (Khan et al., 2007; Sandifer and Hopkin, 1997; Smit and Van Gestel, 1997; Spurgeon et al., 1997). Nevertheless, the present data are unique, because three-factor interactions were studied in a scenario that more accurately represents environmentally realistic conditions. Studies involving three factors with different modes of action are exceptional (Heugens et al., 2006), and no other studies have investigated cumulative effects of three different factors on terrestrial invertebrates.

Substantial differences in the effects of chemicals at different levels were observed. Those beetles, which were able to survive the first few days after CPF application, survived almost as long as the control beetles if they were not exposed to Ni. This probably was because of the fast degradation of CPF, which is a relatively nonpersistent insecticide with a soil-surface half-life of approximately 3-14 days

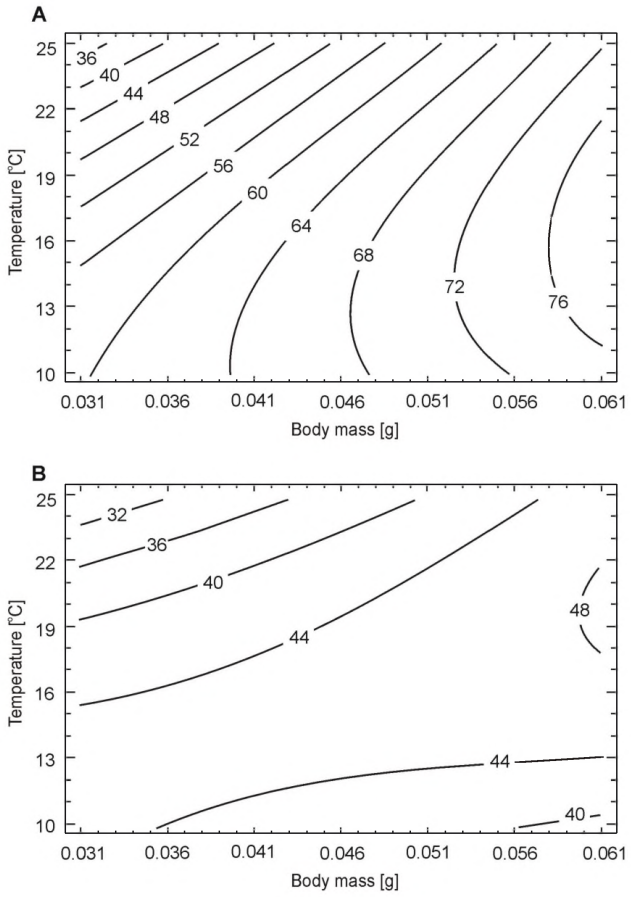

Fig. 3.5. Effect of temperature and body mass on fecundity (ranked number of eggs marked on isoboles) of *Pterostichus oblongopuncatus* at different Ni treatments: isobolic representation of the interaction (p=0.004) between body mass and temperature shown for 0 mg Ni kg^{-1} (A) and 5000 mg Ni kg^{-1} (B). Note that heavier beetles fed uncontaminated food reproduce better than smaller ones irrespective of temperatures, whereas when exposed to 5000 mg Ni kg^{-1} the smaller ones reproduce better at low temperatures and the heavier ones reproduce better at higher temperatures.

(Barron and Woodburn, 1995). In contrast to the pesticide, Ni toxicity was low at the beginning of the experiment, but the mortality gradually increased with increasing exposure time. A similar phenomenon was observed by Laskowski (2001) for cadmium and imidacloprid in aphids and led to the conclusion that in short-term bioassays, the effects of pesticides may be overestimated whereas those of metals would be underestimated. Nickel effect was concentration dependent, resulting in

higher mortality at higher concentration. From a study on Ni kinetics (Bednarska et al., 2007; Chapter 5), it is known that beetles accumulate Ni to a large extent only during the first few days of exposure and, then, are able to start efficient decontamination even if they are still exposed to highly contaminated food. Because physiological mechanisms of metal detoxification probably are costly (Lukasik and Laskowski, 2007), however, less resources may be available for production (growth and reproduction) and/or for maintenance (Sibly and Calow, 1989). Thus, it is possible that even if the beetles did not accumulate a high amount of Ni, they could suffer from its indirect effects. On the other hand, the short period of Ni accumulation observed in a 64-d kinetics study does not necessarily mean the same relationship for longer exposure time and/or different Ni concentrations. In an earlier study, it was showed that after long exposure (245 d), Ni concentration in beetles increased with increasing Ni concentration in food (Chapter 2).

Barron and Woodburn (1995) reported that terrestrial invertebrates are relatively tolerant to CPF exposure, and in some most sensitive species, contact toxicity may occur at concentrations of approximately 100 ng per insect. Indeed, the present results show that at optimal temperature, 80 ng CPF beetle^{-1} caused only a minor, nonsignificant decrease in survival of the beetles fed uncontaminated food. A significant effect, however, was seen when the beetles were exposed simultaneously to Ni and/or different temperatures. This clearly underlines the importance of considering multiple factors in assessment of risks brought by exposure to toxic chemicals. Environmental pollution can reduce the tolerance to climatic factors, and exposure to suboptimal temperatures may enhance negative effects of toxicants. These interactions are of high importance for the viability of populations in the real world (Holmstrup et al., 2000). An increase in sensitivity to drought stress in springtails (*Protaphorura armata*) exposed to pyrene was shown by Sjursen and Holmstrup (2004). Similarly, previous exposure to polycyclic aromatic hydrocarbons (pyrene and fluorine) and a detergent (nonylphenol) increased the susceptibility of the collembolan *Folsomia candida* to drought stress (Sørensen and Holmstrup, 2005), but in that same study, no effect of previous exposure to insecticide (dimethoate) on drought tolerance was

observed. In other studies, the ability of springtails (Holmstrup, 1997) and earthworms (Holmstrup et al., 1998) to tolerate drought was greatly impaired by copper exposure. No general concordance however, exists among different studies regarding the effects of metals at different temperatures. Some authors found a clear effect of temperature on metal toxicity in terrestrial invertebrates (Spurgeon et al., 1997), whereas others did not (Abdel-Lateif et al., 1998). No general trend can be found in the metal-temperature interaction also in the present study, because it seems that different endpoints may be differently sensitive to such interactions. In spite of the longest reproduction period, the total number of eggs laid by females at 10°C was lower than the number of eggs produced at 20°C. It was also observed that the proportion of hatched larvae was much lower in beetles cultured at 10°C than in those cultured at 20°C. Thus, 20°C seems to be optimal for *P. oblongopunctatus* reproduction,even if exposed to Ni. In summary, it was found that reproduction was most sensitive to Ni concentration at the lowest and highest temperature, whereas survival after exposure to Ni decreased with increasing temperature.

Stone et al. (2001) showed that carabids inhabiting chronically polluted environments were less tolerant to additional environmental stressors (pesticide or starvation) compared with those from uncontaminated areas. On the other hand, long-term exposure to high levels of Ni enabled *C. pardalina* to cope with other stressors, including short-term exposure to OP dimethoate (Augustyniak et al., 2007). Results of the studies presented herein are in agreement with those obtained by Stone et al. (2001), because exposure to a mixture of the two chemicals resulted in significant positive interaction observed for beetles survival, indicating higher effect than expected from the simple sum of effects of single toxicants. Additionally, the three-factor interaction (CPF×Ni×T) showed that the beetles were less sensitive to combined effect of the chemicals at lower temperatures than at higher temperatures. In ectotherms, the metabolic rate increases with temperature, which leads to increased consumption and, in consequence, may enhance assimilation of toxicants from food and intoxication of exposed animals. On the other hand, because an increase in temperature generally increases the rate of all metabolic processes, an organism can,

theoretically, detoxify and/or eliminate toxicants more effectively at elevated temperatures. Thus, the final outcome, observed as internal body concentration and effects of intoxication, would depend on the balance between these two effects under different temperatures: the consumption and assimilation rates of toxicants on the one hand, and the detoxification and elimination rates on the other. In the present study Ni levels in the beetles cultured at different temperatures were not measured, because Ni-contaminated food was present in the guts of dead animals, which would influence the results. Nevertheless, a study on Ni kinetics (Bednarska et al., 2007; Chapter 5) done at five different temperatures (including those examined in the present study) did not demonstrate significant differences in the uptake and elimination rates between temperatures. Thus, the increase in Ni effect with increasing temperature probably is not caused by an elevated Ni accumulation but, rather, by effects on the metabolic rate of beetles. Similarly, Donker et al. (1998) concluded that greater toxicity of zinc to *Porcelio scaber* at higher temperature was caused by an increase in isopods metabolism rather than by increased zinc accumulation.

The lower sensitivity of beetles to joint effect of Ni and CPF at 10°C than at 25°C also may be connected with increased toxicity of CPF at higher temperature. As indicated by the study of Lydy et al. (1999) on *Chironomus tentans*, the toxicity of OP pesticides can be strongly affected by changes in abiotic factors, such as temperature. Temperature influences the physical and chemical state of toxicants, and increased temperature may accelerate biotransformation of the CPF into more toxic metabolites (Lydy et al., 1999). This trend is clear also for other species and pesticides. For example, Satpute et al. (2007) found that CPF was most toxic for the moth *Earias vitella* at higher temperatures. Similar results were reported by Brecken-Folse et al. (1994) for an aquatic invertebrate, the grass shrimp (*Palaemonetes* spp.), exposed to two OP insecticides (terbufos and trichlorfon). Additionally, the metabolic activation of OPs may be also enhanced by the presence of metals (Forget et al., 1999), which may explain the stronger effect on survival when beetles are exposed to both pollutants simultaneously. The mechanisms by which the negative effect of a toxicant is enhanced by the presence of other pollutants may result as well as from disturbances in

metabolic activities of cytochrome P450 toward insecticides, inhibition of detoxifying systems, or an interaction of both processes (Bone and Chambers, 1997). In fact, Forget et al. (1999) showed that combination of OPs with metals (arsenic and cadmium) had a synergistic effect on inhibition of AChE activity in the microcrustacean *Tigriopus brevicornis*. However, because Ni can generate oxidative damage to DNA and cause lipid peroxidation (Eisler, 1998), whereas the main target point for CPF is AChE, it also is possible that the effect of the binary mixture of chemicals on beetles survival resulted from accumulation of single negative effects specific for each chemical. Because no studies dealing with effects of CPF in combination with Ni under temperature stress were found, the explanation of biochemical mechanisms behind Ni and CPF toxicity and their interactions at different temperatures needs further research.

The observation that even though the effect of CPF generally was negative, the heavier animals were able to survive longer after the pesticide exposure (significant interaction between body mass and CPF) is in accordance with expectations. Because the CPF dose was the same for all individuals, the heavier animals received smaller dose per unit mass. Also, when exposed to Ni or high temperature, the heavier beetles reproduced better (significant interactions, mass×Ni and mass×T). The mass×Ni interaction was significant across the whole experiment, whereas the latter was found only in beetles fed with uncontaminated food. In fact, for beetles fed 5000 mg Ni kg^{-1}, a different relationship was found, indicating better reproduction of heavier beetles, but only at higher temperatures; at low temperatures, the smaller beetles reproduced better. It must be noted, however, that the low number of animals that entered the reproduction part of the experiment, especially at the highest Ni concentration, reduced the statistical power behind this notion, which probably caused the results that neither Ni nor CPF as single factors turned up as significant in the final equation describing egg production. Moreover, the conclusions from the reproduction data might be disturbed by naturally high variability in the numbers of eggs produced by this species, as observed in the present and previous studies (Łagisz et al., 2002).

The data obtained in this experiment indicate a rather complex relationship between chemicals and natural environmental factors, such as temperature, and show that the interactions between toxicants representing different classes of chemicals may be different at different temperatures. Such differences in the toxicity of chemicals at different temperatures clearly show that ecological risk assessment cannot rely on simple ecotoxicological tests performed under near-optimal conditions, which favor high survival and reproduction in test organisms. Such tests may lead to underestimation of pollutant impact on field populations, which regularly encounter several physical and chemical stressing factors simultaneously.

Results of the present study indicate that multiple stressors, such as metals, pesticides, and extreme temperatures, can lead to outcomes that are not possible to predict when studying each stressor separately. The significant and complex interactions among the chemicals and temperature indicate that even if interactive effects sometimes are difficult to interpret, an urgent need exists to study such interactions if laboratory-generated toxicity data are to be extrapolated to the field conditions.

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CHAPTER 4

ENVIRONMENTAL CONDITIONS ENHANCE TOXICANT EFFECTS IN LARVAE OF THE GROUND BEETLE, *PTEROSTICHUS OBLONGOPUNCTATUS*

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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. Studies presented in Chapter 3 demonstrated that combined effect of nickel (Ni) and chlorpyrifos (CPF) 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, the effects of the same factors on the beetle larvae were studied. 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×T interaction on the proportion of emerged imagines indicated stronger toxicity of Ni at 25°C than at 10°C.

Keywords: multiple interactions, *Pterostichus oblongopunctatus*, metals, pesticides, temperature

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 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 (Donker et al., 1998; Spurgeon et al., 1997; Chapter 2), but there is a lack of research focusing on interactions of mixtures of dissimilar chemicals at different temperatures. Previous work (Chapter 3) 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, the experiment with continuous exposure to Ni through food and with a single application of CPF to the soil was designed. As in previous study on adults (Chapter 3), 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 Ni on soil-dwelling invertebrates has increased in recent years as production of the metal has risen (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*, no other data on Ni and CPF interaction for soil invertebrates have been found. 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.

Materials and Methods

Pterostichus oblongopunctatus *culture*

The larvae for the experiment were taken from approximately 100 adult beetles collected from an uncontaminated forest near Krakow, southern Poland. The beetles were cultured to obtain larvae as described in Chapter 2. 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, these two temperatures were taken as the minimum and maximum, and 20°C as optimal (cf. also Metge and Heimbach, 1998).

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 approximately 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 (7:3 dry weight), with 1 g sodium benzoesate ($C_7H_5NaO_2$; Fluka, Deisenhofen, Germany) per kg 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 surface. Thus, it was assumed that shortly after spraying, the highly toxic but gradually degrading CPF is toxic to soil-dwelling larvae mostly through soil rater 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 to 18 replicates used for each treatment. Altogether, 492 larvae were used in the experiment. Nickel chloride hexahydrate ($NiCl_2 \times 6H_2O$, Eurochem BGD, Warsaw, Poland) was added to the food as aqueous solution, and CPF (minimum purity, 98%; technical grade; Cheminova, Lemvig, 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 2nd day to ensure that effects observed in adults resulted from exposure in 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.

Chemical analyses

To determine the actual Ni concentrations in food, a dry sample approximately 0.1 g (WPA 180/k Radwag, Radom, Poland), Poland of each batch was analyzed by flame atomic absorption spectrometry (AAnalyst 800; Perkin-Elmer, Boston, MA, USA). At least three blanks accompanied every run of analysis. Additionally, samples of certified reference material (dogfish muscle DORM-2; National Research Council, Ottawa, ON, 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 mg kg^{-1} dry weight.

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 approximately linear relationship between temperature and both survival and proportion of emerged imagines, the following model was tested:

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

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 nonsignificant terms were consecutively removed from the model, starting with those with the highest p value, as long as only significant factors ($p \leq 0.05$) remained.

All analyses were done using Statgraphics Centurion XV (Statpoint Inc.; http://www.statgraphics.com).

Results

The measured Ni concentrations in food were in good accordance with the assumed nominal values. The average concentration of Ni in control food was 10.6 mg kg^{-1} dry weight (± 4.2, SD), and 612 (± 54.8) and 1145 (± 364) at nominal treatments 600 and 1200 mg kg^{-1}, respectively. No significant effect on survival due to the use of acetone as a solvent was found at any temperature (p values at least 0.1).

Survival

The survival curves for all exposure conditions differed significantly ($p<0.0001$) and were analyzed consecutively within each stressor (Ni, CPF or T). Figure 4.1 shows survival of beetles exposed to various Ni concentrations at each combination of temperature and CPF (3×4). Table 4.1 gives the median survival times (LT_{50}) for all treatments but one in which mortality during the experiment was not high enough (Ni-0/CPF-1/T-10). Nickel dosed at 1200 mg kg^{-1} significantly decreased survival in all CPF treatments at 10°C ($p\leq0.04$) versus the same treatments without Ni. Also at 10°C, larvae fed Ni at 600 mg kg^{-1} had significantly higher mortality than those fed uncontaminated food at CPF-1, but had better survival than those fed 1200 mg Ni kg^{-1} at both CPF-0 and CPF-1. At 20°C the only significant differences in survival curves between Ni concentrations were at 1 mg CPF kg^{-1} ($p=0.006$): the mortality of larvae fed either 600 or 1200 mg Ni kg^{-1} was significantly higher than that of those fed uncontaminated food. At 25°C there were no differences in survival between Ni concentrations at any CPF concentration, but the LT_{50} values were lower than for the corresponding treatments at 10°C and 20°C (Tab. 4.1). The larvae from 10°C had the longest median survival time at all Ni/CPF treatments. The differences in survival between temperatures were significant ($p\leq0.02$) at all combinations of Ni with CPF. For CPF analyzed for its effect as a single toxicant there were no differences in survival between the concentrations at 10°C regardless of the Ni concentrations, but both CPF-1 and CPF-2 significantly increased mortality ($p\leq0.02$) versus CPF-0 treatment at all Ni concentrations at both 20 and 25°C. The mortality of larvae treated with CPF-0.5 was lower ($p<0.001$) than at CPF-2 in Ni-1200/T-20 and at both Ni-600/T-25 and Ni-1200/T-25.

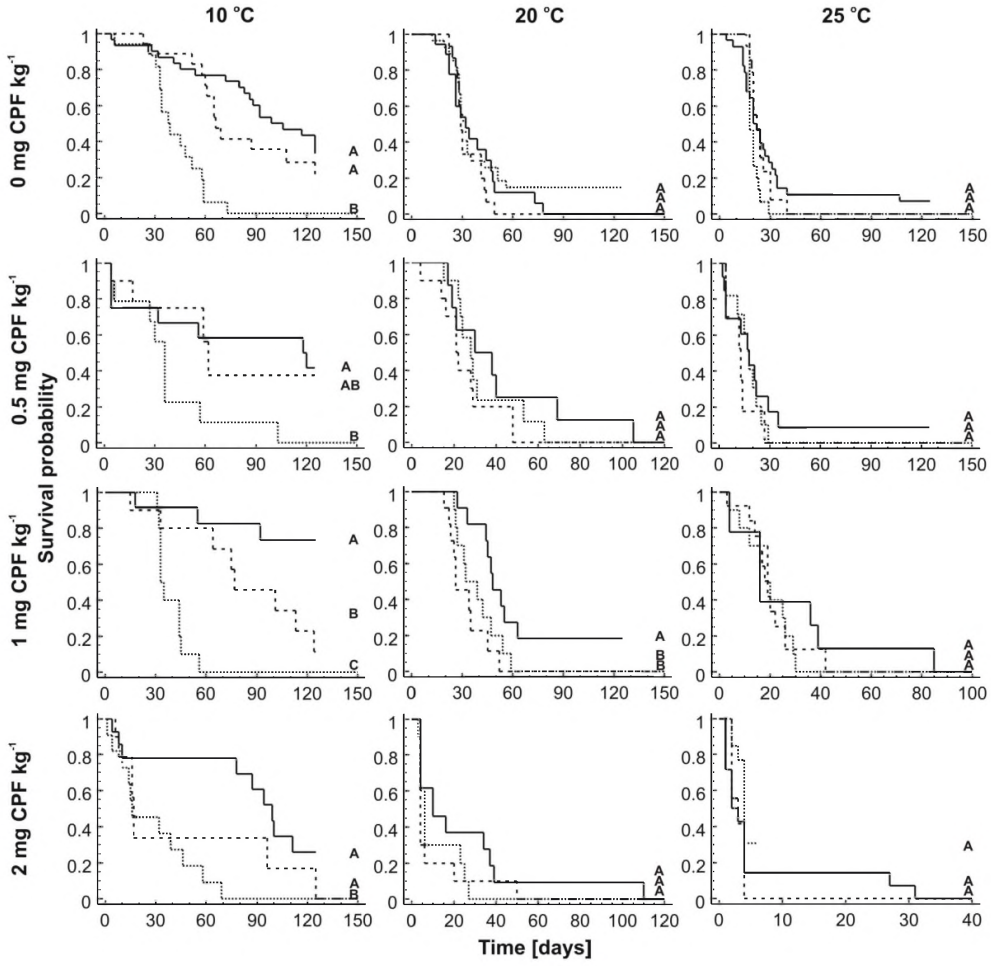

Fig. 4.1. Survival of *Pterostichus oblongopunctatus* larvae exposed to various Ni concentrations in food (— 0, – – 600, ▪ ▪ ▪ 1200 mg Ni kg^{-1}) at 10, 20 or 25°C and 0, 0.5, 1 or 2 mg CPF kg^{-1} food as a function of time. The same letter means no significant differences in lifetime between Ni treatments.

Table. 4.1. Median survival times (LT_{50}) for *Pterostichus oblongopunctatus* larvae (median ± SE); number of individuals in brackets.

		Median survival time in days		
Treatment		Temperature [°C]		
Ni [mg kg^{-1}]	CPF [mg kg^{-1}]	10	20	25
0	0	106±15.8 (31)	32±1.9 (31)	22±2.2 (30)
	0.5	118±55.4 (12)	30±12.0 (12)	18±4.1 (13)
	1	– (12)	48±4.4 (12)	16±5.3 (10)
	2	99±9.9 (14)	10±6.7 (13)	3.0±0.9 (14)
600	0	66±4.1 (13)	32±5.4 (18)	22±2.3 (16)
	0.5	62±3.3 (10)	21±3.2 (10)	13±4.9 (10)
	1	77±18.0 (10)	26±4.6 (11)	19±1.7 (13)
	2	17±0.7 (10)	4.0 (10)	3.0±1.2 (10)
1200	0	39±4.9 (17)	29±1.0 (17)	18±0.8 (17)
	0.5	36 ± 2.5 (10)	28±3.6 (11)	18±2.3 (11)
	1	33±1.2 (10)	32±6.3 (10)	19±4.2 (10)
	2	16±9.9 (11)	6.0±1.0 (10)	4.0±0.3 (13)

The GLM analysis indicated that Ni and T as well as the interactions CPF×Ni, CPF×T and CPF×Ni×T had a marked effect on survival of larvae (Table 4.2). The final model (that is, the one with significant variables and interactions only) was significant at $p<0.0001$ and explained 45% of total variance in the life span of *P. oblongopunctatus* (adjusted R^2=44.4%). The significant interaction between CPF and Ni (p=0.005) indicates that the toxicity of one chemical was affected by the presence of the other. Temperature affected CPF toxicity ($p<0.0001$) but to a lesser extent at lower than at higher Ni concentration, as can be seen from Fig. 4.2 showing the lifetime isoclines for beetles treated with different combinations of CPF and temperatures at two extreme Ni

concentrations: 0 and 1200 mg kg^{-1}. The three-factor interaction between the two pollutants and temperature was significant at p=0.01.

Table 4.2. Type III sums of squares and significance levels of the variables for the final general linear models for ranked lifetime of *Pterostichus oblongopunctatus* treated with Ni and CPF at different temperatures (T)*.

Model term	Sum of squares	*df*	Mean square	*F*-ratio	*p*-value
Ni	69941.1	1	69941.1	5.92	0.02
T	1.335×10^{-6}	1	1.335×10^{-6}	113.06	<0.0001
CPF×Ni	87403.4	1	87403.4	7.40	0.005
CPF×T	643917.0	1	643917.0	54.53	<0.0001
CPF×Ni×T	76488.6	1	76488.6	6.48	0.01
RESIDUAL	4.983×10^{-6}	422	11808.0		
TOTAL (CORRECTED)	9.167×10^{-6}	427			

* Only significant variables are included, *df* = degrees of freedom

Proportion of emerged imagines

Except for the Ni-1200/CPF-1/T-20 treatment, in which one adult beetle was found, no imagines emerged from larvae treated with 1200 mg Ni kg^{-1}; this is because many of those larvae died or failed to moult to the pupal stage. For the same reasons, no imagines emerged from larvae fed 600 mg Ni kg^{-1} at the two higher CPF concentrations at 20°C and at the highest CPF concentration at 10°C. Only five adults emerged from larvae cultured at 25°C: three from the control treatment (Ni-0/CPF-0), one from Ni-0/CPF-0.5, and one from Ni-0/CPF-1. The proportion of emerged imagines in most Ni/CPF treatments at 10°C was higher than in the corresponding Ni/CPF treatments at 20°C, but the larvae cultured at 10°C needed more time to reach imago stage than those at 20°C.

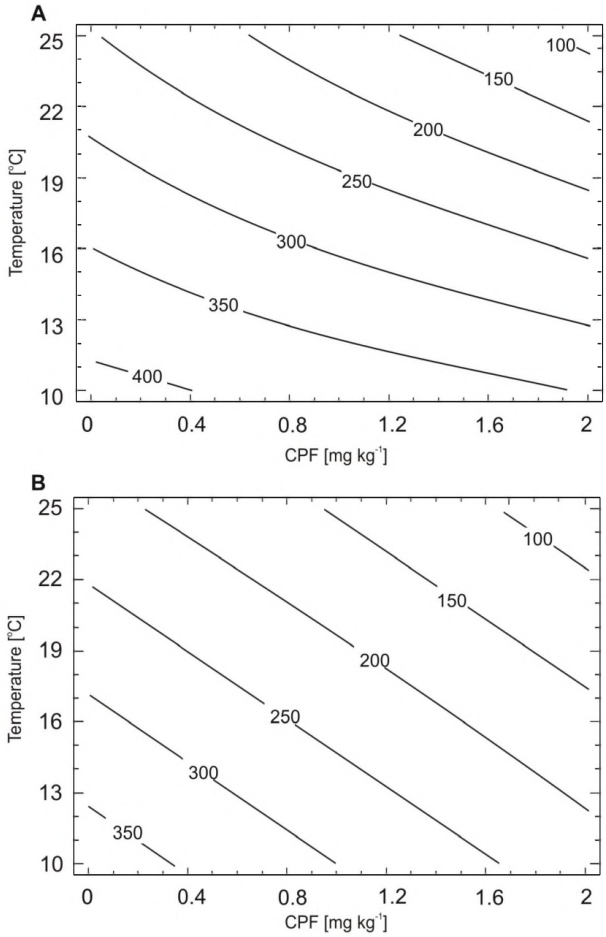

Fig. 4.2. Temperature-dependent effect of CPF on lifetime (ranked number of days marked on isoboles) of *Pterostichus oblongopuncatus* larvae – isobolic representation of the interaction between CPF and temperature shown for beetles not exposed to Ni (A) and exposed to 1200 mg Ni kg^{-1} (B). Note that the interaction depends on Ni treatment, as indicated by a significant three-factor interaction, Ni×CPF×T (p=0.01).

Ni and CPF treatments led to a significant decrease of the proportion of emerged imagines (p=0.0001 and p=0.01, respectively). The proportion of emerged imagines also depended on temperature (p=0.003). Temperature strongly modified the effect of Ni (p=0.03): emergence was lower for Ni-exposed larvae at higher than at low temperature (Tab. 4.3, Fig. 4.3).

Table 4.3. Type III sums of squares and significance levels of the variables for the final general linear models for ranked proportion of emerged imagines of *Pterostichus oblongopunctatus* treated with Ni and CPF at different temperatures (T)*.

Model term	Sum of squares	*df*	Mean square	*F*-ratio	*p*-value
CPF	341.0	1	341.0	7.52	0.01
Ni	918.9	1	918.9	20.25	0.0001
T	756.8	1	756.8	16.68	0.0003
Ni×T	240.0	1	240.0	5.29	0.03
RESIDUAL	1406.7	31	45.4		
TOTAL (CORRECTED)	4949.2	35			

* Only significant variables are included, *df* = degrees of freedom

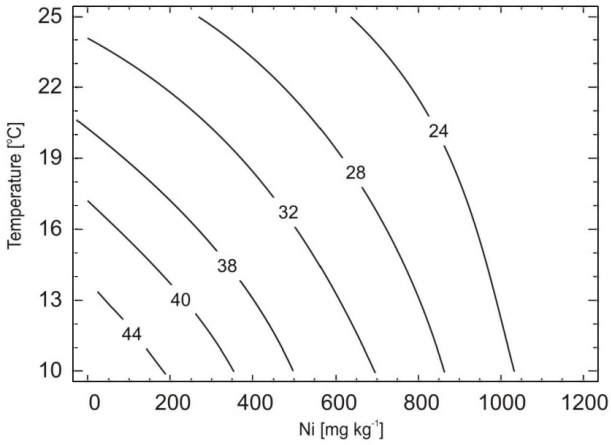

Fig. 4.3. Effect of temperature and Ni on proportion of emerged imagines (ranked number of emerged imagines marked on isoboles) of *Pterostichus oblongopuncatus* larvae – isobolic representation of the interaction (p=0.03) between Ni and temperature shown for beetles not exposed to CPF.

Discussion

Nickel has recently become a serious pollutant (Kabata-Pendias and Mukherjee, 2007) as Ni processing emissions have raised its environmental concentrations (Eisler, 1998). The background levels usually range from 20 to 40 mg kg^{-1} soil (Kabata-Pendias and Mukherjee, 2007), but around smelters may reach extremely high values from, for example, 6400 mg Ni kg^{-1} (Stefanowicz et al., 2008) up to 22,000 mg Ni kg^{-1} (Everhart et al., 2006). Taking the high Ni concentrations observed in smelting-contaminated soils together with the likelihood that studied beetles may require Ni at somewhat elevated levels (Bednarska and Laskowski, 2008), the concentrations used in the present study are in the range of environmentally realistic concentrations for polluted areas. Typical agricultural soil applications of CPF result in soil surface residues of 0.3 to 32 mg kg^{-1} (Racke et al., 1994).

The 1200 mg kg^{-1} Ni dose proved highly toxic to larvae: only a single imago (at CPF-1/T-20) emerged at that concentration. Similarly, beet armyworm *Spodoptera exigua* larvae fed an artificial diet spiked with 900 mg Ni kg^{-1} reached pupa stage half as frequently as the controls (Zawisza-Raszka and Dolezych, 2008). While Ni treatment decreased larva survival especially at lower temperatures, CPF exerted significant effects on larva survival at 20 and 25°C, but not at 10°C. Apparently, low temperature protected the larvae against the adverse effect of the pesticide, as can also be seen from the CPF×T interaction. Probably at low temperature the biotransformation of CPF into more toxic metabolites was reduced, as earlier observed for CPF and other OP pesticides (Brecken-Folse et al., 1994; Lydy et al., 1999; Satpute et al., 2007).

Chlorpyrifos, like many other OPs, is highly toxic to target organisms (pests), but may also harm beneficial species, including carabid beetles (Cox, 1995). Chlorpyrifos is reported to be toxic to soil-dwelling insects at concentrations of 0.5–5 mg kg^{-1} (Fountain et al., 2007). It is a relatively nonpersistent broad-spectrum insecticide with a soil surface half-life of about 3-14 days (Barron and Woodburn, 1995), although the half-life may range from a week to 24 weeks depending on soil moisture, microbial activity, organic matter content and temperature (Odenkirchen and Eisler, 1988). In the

carabid beetle *Poecilus cupreus*, Heise et al. (2005) showed decreased dimethoate-induced larvae mortality with increasing soil organic carbon content. This might be due to binding of the pesticide to soil colloids, possibly reducing its bioavailability. In the present experiment the larvae were kept in wet peat rich in organic matter, but at the collection site the organic C content of the humus layer is less than 16% (Stefanowicz et al., 2008). Thus, it should be expected that the effects of CPF on larvae of *P. oblongopunctatus* in their natural environment would be more severe than those observed in the present study. In the field, CPF can affect carabid beetles not only via direct toxicity but also through indirect effects (e.g., a scarcity of edible prey). Indeed, some researchers have shown that the richness and diversity of springtails, an important prey of *P. oblongopunctatus* (Brunsting, 1981), can be negatively affected by the application of an insecticide such as CPF (Fountain et al., 2007; Frampton and Van den Brink, 2007). Outdoors, carabids may also suffer lethal effects by consuming pesticide-contaminated prey (Mauchline et al., 2004).

The proportion of emerged imagines was highest at 10°C, but development of larvae raised at that low temperature took longer than at 20°C. This result is in accordance with the common observation that higher temperature accelerates physiological processes in ectotherms. Because the metabolic rate goes up with temperature, metals and/or pesticides may act more rapidly in the cells, so death may occur sooner. On the other hand, accelerated metabolism may allow more efficient detoxification and excretion of chemicals. While some insights about the interaction between Ni and temperature can be drawn from studies on aquatic organisms (Bryant et al., 1985), little is known about such effects in soil invertebrates. In earlier work no interactive effect of Ni treatment and temperature for the metabolic rate of *P. oblongopunctatus* was found (Bednarska and Laskowski, 2008). Ni is a rather poorly accumulated metal (Kabata-Pendias and Mukherjee, 2007). The beetles can eliminate this metal efficiently even if exposed to high concentrations in food, and in earlier work on this species no temperature-caused differences in Ni elimination rates were found (Bednarska et al., 2007; Chapter 5). This suggests that the high-level interaction CPF×Ni×T on larva survival was not due to the influence of temperature on Ni toxicity as such, but rather

the influence of temperature and/or Ni on CPF toxicity. Two two-factor interactions found for larva survival support this presumption: CPF×Ni and CPF×T. On the other hand, the synergistic Ni×T interaction found for the proportion of emerged imagines implies that the formation of a pupa and/or emergence from it may be subject to factors other than larva survival.

As indicated by studies on *P. oblongopunctatus* (Chapter 2) and *Folsomia candida* (Smit and Van Gestel, 1997), different life traits such as survival and reproduction may be differently sensitive to toxicants. Similarly, different life stages of beetles may not be equally sensitive to toxicants. Work by Łagisz et al. (2002), for example, demonstrated changes in population parameters of adult *P. oblongopunctatus* under chronic multigenerational exposure to toxic metal concentrations, while Mozdzer et al. (2003) noted no changes in the larval stage of this species. On the other hand, in the earlier (Chapter 3) and present studies it was demonstrated that interactions between CPF and Ni were temperature-dependent in both life stages. Moreover, the synergistic two-factor interaction between Ni and CPF found for larvae strengthens the hypothesis that exposure to one chemical reduces tolerance to another, and supports the findings of Forget et al. (1999) and Stone et al. (2001). Forget et al. (1999) showed synergistic lethal effects of a series of combinations of a metal (arsenic, copper, cadmium) and a pesticide (carbofuran, dichlorvos, malathion) for copepods *Tigriopus brevicornis*, while Stone et al. (2001) found that *P. oblongopunctatus* originating from areas highly polluted with metals are more susceptible to additional exposure to dimethoate or starvation.

Conclusions

The present and previous studies (Chapter 3) showed that both larvae and adults of *P. oblongopunctatus*, a species belonging to a group very important to soil ecosystem functioning, are good indicators of multiple stressor effects, giving consistent results for both stages. Those results indicate similar interactive effects on survival, through different exposure routes. To better understand the effects of multiple stressors in the

environment and to integrate this knowledge into ecological risk assessment, this line of research needs to be continued.

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CHAPTER 5

NICKEL TOXICOKINETICS UNDER THE INFUENCE OF TEMPERATURE AND CHLORPYRIFOS IN THE GROUND BEETLE *PTEROSTICHUS OBLONGOPUNCTATUS* (COLEOPTERA: CARABIDAE)

This chapter has been submitted in modified forms as:

Bednarska AJ, Brzeska, A. Laskowski R. Unexpected toxicokinetics of nikel in the ground beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae): the influence of temperature on nickel toxikokinetics.

Abstract

Nickel kinetic was studied in the ground beetle *Pterostichus oblongopunctatus* additionally exposed to different, potentially stressful, temperatures and to the organophosphate insecticide chlorpyrifos (CPF). An unexpected Ni kinetics in metal-exposed adult and larval beetles were found: instead of the pattern observed commonly for other metals, that is, an increase in metal concentration followed by stabilisation in the uptake phase and a decrease after transfer to uncontaminated food, the Ni-fed beetles apparently switched to decontamination soon after the start of Ni exposure, while they were still being fed Ni-contaminated food. Also, internal body Ni concentrations showed very high variance. The traditional first-order one-compartment model with the switch to decontamination set to the last day of the uptake phase appeared inadequate and in most cases was nonsignificant. Instead, the model with a regression-estimated point of switching to decontamination fit the data better, explaining 54 - 91.5% of the temporal variability of mean Ni body concentrations (weighted regression). In fact, the pattern of the data suggests a three-phase model, but a larger data pool is needed for this hypothesis to be tested statistically. Temperature did not affect Ni toxicokinetics in adults, but in larvae there were some temperature-dependent differences in kinetic parameters. Neither assimilation nor elimination of Ni were affected by CPF suggesting that their actions in combination are independent.

Keywords: toxicokinetics, metals, pesticides, temperature

Introduction

Studies on metal kinetics in animals are of particular importance in ecotoxicology as the accumulation rate rather than absolute concentrations of metals is a key predictor of toxicity (Van Straalen et al., 2005). A number of studies on assimilation and elimination of different metals have been done in terrestrial invertebrates (e.g., Bibič et al., 1997; Janssen et al., 1991; Kramarz 1999ab; Nauhauser et al., 1995; Spurgeon and Hopkin, 1999; Sterenborg et al., 2003; Witzel, 2000; Vijver et al., 2005), including the carabid species *Notiophilus biguttatus* (Janssen et al., 1991), *Pterostichus niger* (Lindquist et al., 1995) and *Poecilus cupreus* (and Kramarz, 1999a). In all those studies the animals were exposed to elevated concentrations of a metal (uptake phase), followed by the elimination phase when they were transferred to uncontaminated test substrate. Analysis of metal concentration changes in animals during both phases has provided information on the rate of metal assimilation and excretion, the possibility of removing the excess of metal from the body, and the existence and level of an equilibrium concentration, that is, the metal concentration that does not change with time any longer because the assimilation rate is balanced by excretion. Thanks to that work, a range of diverse metal accumulation and elimination strategies have been recognized. In some studies, during the uptake phase the xenobiotics (e.g., Cd, Pb) showed a very rapid increase in concentration followed by a plateau; than there was a rapid decrease after transfer to uncontaminated medium, leading to almost complete depuration from metal excess, whereas concentrations of essential metals (e.g., Zn, Cu) were maintained at almost constant level (Kramarz, 1999ab; Janssen et al, 1991). Such differences in kinetic patterns might be expected, as nutritional metal concentrations are regulated more efficiently than those of xenobiotic metals. In their study of the earthworm *Eisenia fetida*, however, Spurgeon and Hopkin (1999) found that for Cd and Pb the equilibrium level was not reached during the uptake phase, and only slow excretion was noted after the worms were transferred to clean soil, whereas Zn exhibited fast initial assimilation followed by equilibrium after a few days of exposure, and rapid excretion after transfer to clean soil. Such interspecific in the toxicokinetics of the same metals led Hopkin (1989) to observe that assimilation and elimination rates

of metals in invertebrates depend not only on the biological role of a metal (essential vs nonessential) but also on he physiology of the species. Janssen et al. (1991) compared Cd kinetics in four species of soil arthropods, and confirmed that species-specific differences in metal detoxification are important in determining the metal kinetics patterns. They showed that predators (carabid *Nothiopilus biguttatus* and pseudoscorpion *Neobiscum muscorum*) had higher Cd assimilation rates than saprotrophic species: (springtail *Orchesella cincta* L. and oribatid mite *Platynothurus peltifer* Koch), but the excretion rates and equilibrium concentrations were related to taxonomic rather than trophic position. Due to differences in detoxification mechanisms, insects excreted Cd more efficiently and maintained lower equilibrium concentrations than arachnids, in which the detoxification process is based on storage of metals (Janssen et al., 1991). In her studies on *Poecilus cupreus* (1999a) and *Lithobius mutabilis* (1999b), Kramarz also showed metal assimilation and excretion patterns to be strictly related to organism's detoxification mechanisms. Other factors such as the concentration of a metal (Bibič et al., 1997; Heikens et al., 2001), animal feeding rate (Bibič et al., 1997; Janssen et al., 1991) and/or abiotic factors such as soil pH (Sandifer and Hopkin, 1996; Janssen et al., 1997; Vijver et al., 2007) and temperature (Janssen and Bergena, 1991; Spurgeon et al., 1997) may also influence the dynamics of metal accumulation and/or excretion, and thus their toxicity.

Temperature is a natural factor of major importance to the physiological state of an organism, and it is highly variable in the field. In poikilotherms, acceleration of metabolic rates at high ambient temperature might increase the assimilation and consequently the toxicity of metals; or on the other hand, it might allow more efficient detoxification. Without detailed experimental studies it seems difficult if not impossible to predict the final effect of temperature on metal toxicokinetics. Although it has been more than thirty years since it was suggested that studies on the effect of temperature on assimilation and excretion would aid our understanding of toxicant kinetics (Berger and Dallinger, 1989), few data are available on terrestrial invertebrates in this regard (Janssen and Bergena, 1991).

At many contaminated sites, various toxic chemicals may interact and increase or decrease each other toxicity. Whether these effects are caused by changes in the toxicokinetics of interacting chemicals remains largely unknown. There are some data on the effects of one metal on the toxicokinetic of another. For example, Sterenborg et al. (2003) found that Zn decreased the Cd assimilation rate in the springtail *Orchestella cincta* exposed simultaneously to both metals. The combination of Zn with high a high Cd concentration completely changed the accumulation patterns for both metals in *Porcelio scaber* (Witzel, 2000), and influenced each other's kinetics in centipedes *Lithobius mutabilis* Koch (Kramarz, 1999b), but not in *Poecilus cupreus* (Kramarz 1999a). No interaction between Zn and Cd was found in *Pterostichus oblongopunctatus* (Lagisz et al., 2005). No toxicokinetics studies involving chemicals with different modes of action in terrestrial invertebrates were found.

The data on nickel kinetics in terrestrial invertebrates are also scarce. The few existing studies of it in various species of earthworms (Neuhauser et al., 1995; Janssen et al., 1997; Peijnenburg et al., 1999) indicated species-dependent assimilation of nickel. Because the data on Ni kinetics in invertebrates are so limited, it is hard to tell whether the body concentration of this metal is actively regulated and therefore perhaps less influenced by temperature, or is not regulated and thus more responsive to temperature. At the same time, the mechanisms of interactions between Ni and chlorpyrifos (CPF) in the ground beetles *Pterostichus oblongopunctatus* found in previous studies (Chapters 3 and 4) remain unclear. In this study, then, the aim was to investigate Ni kinetics in *P. oblongopunctatus* exposed to metal-contaminated food at different temperatures and under the influence of low and high levels of CPF.

Most work describing the uptake and elimination kinetics of different metals in various species is based on the one-compartment model (e.g., Gimbert et al., 2006; Janssen, 1991; Kramarz, 1999ab; Spurgeon and Hopkin, 1999; Sterenborg et al., 2003; Vijver et al., 2006). This model considers an animal as a homogeneous system with a constant assimilation rate and single excretion rate (Atkins, 1969). Such an approach was applied here as well. However, results obtained in this study and detailed analyses of literature data (e.g. Descamps et al., 1996; Lagisz et al., 2005; Nauhauser et al., 1995;

Spurgeon and Hopkin, 1999) showed that the model does not satisfactorily describe metal toxicokinetics in a number of cases (a clear initial fast concentration increase was evident, followed by a decrease well before the animals were transferred to uncontaminated food). This leads to the hypothesis that when suddenly exposed to a highly elevated concentration of a metal, an animal needs some time to adjust its assimilation/decontamination mechanisms to this new environmental situation. This creates a lag time in starting efficient decontamination and/or causes a decrease in the metal assimilation rate, which would explain the toxicokinetic pattern not expected from the traditionally used two-phase model. Therefore, modified toxicokinetic models were also used to describe the behaviour of Ni in *Pterostichus oblongopunctatus* (Coleoptera: Carabidae).

Materials and Methods

Test organism

The ground beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae) is widely distributed in European woods and meadows and can be considered a typical representative of the epigeic carnivorous insects. Ground beetles as a group are important for biodiversity of communities and also for their effectiveness in consuming prey insects, many of which are pest herbivores.

Adult beetles for the study were collected with pitfall traps (plastic cups, ca. 200 ml) from a Scots pine forest in an unpolluted area near Krakow, southern Poland, during two weeks in April 2006 (Experiment I) and April 2007 (Experiment III). They were kept for three weeks in a climatic chamber at 20°C and 75% relative humidity (RH) under 16:8 h L:D, with light intensity 500 lx during the day. The beetles were kept in 1000 ml plastic boxes, 10 individuals per box; the boxes had perforated lids and contained ca. 2 cm peat moistened to at 80% of maximum water-holding capacity (WHC), pH_{H_2O} 4.5-5.0. During this period the beetles were fed uncontaminated artificial food *ad libitum* made of ground mealworms *Tenebrio molitor* mixed with ground apple (7:3 dry weight) with 1 g sodium benzoate ($C_7H_5NaO_2$, Fluka, Deisenhofen Germany) per kg food as preservative (see Chapter 2 for more details).

Before using the beetles in the kinetics study they were weighed to the nearest 0.001 g (WPA 180/k Radwag, Radom, Poland) and placed individually in 30-ml plastic vials filled ¾ with moist peat (pH_{H_2O} 4.5 – 5.0, 80% WHC).

The larvae for the Experiment II were taken from ca. 100 adult beetles collected in 2006. The beetles were cultured to obtain larvae as described in Chapter 2. The newly hatched larvae were transferred individually to 30 ml plastic vials filled ca. 3/4 with moistened peat (80% WHC) with a 3 cm deep hole made with a needle. The larvae were kept in darkness at 20°C and 75% RH. One-day-old larvae from a two-week collection period were randomly assigned to particular treatments and used in the experiment.

Studied factors and food contamination

Temperatures 10, 15, 20, 22.5 and 25°C were chosen for Experiments I and II based on results from an earlier range-finder study, and nickel (Ni) and chlorpyrifos (CPF) were used as test chemicals. The former represents common industrial pollutants heavy metals; the latter is one of the most commonly used pesticides in Europe. Due to their widespread occurrence, it is highly probable that in some areas organisms are exposed simultaneously to both chemicals or other representatives of the two groups. Nickel chloride ($NiCl_2 \times 6H_2O$, Eurochem BGD, Warsaw, Poland) was added to the dry food as aqueous solution at 2500 mg Ni kg^{-1} food in the experiments on adults, and at 300 mg Ni kg^{-1} in the experiment on larvae. Chlorpyrifos (min. 98% technical, Cheminova, Denmark) was added to the dry food as acetone solution and the acetone was evaporated overnight. Controls with and without acetone were included. The food was moistened to 50% of its previous water content and was frozen at -20°C. The animals were fed *ad libitum* every second day; a new batch of unfrozen food was used each time. When fresh food was supplied, the remains of the old food were taken out to keep the boxes and the vials as clean as possible.

Experimental design

Experiment I: Effects of temperature on Ni toxicokinetics in adult beetles

The beetles were randomly allocated to five experimental groups differing only in ambient temperature (10, 15, 20, 22.5 or 25°C). One week later, at least 50 beetles from each temperature group were randomly assigned to the Ni treatment group and, due to the limited number of beetles, 20 beetles only from 10, 15 and 20°C were assigned to controls. The beetles were then fed for the next 64 days with food either contaminated with nickel at 2500 mg Ni kg^{-1} (uptake phase) or clean (control). After 64 days, Ni-exposed beetles were transferred to uncontaminated food (elimination phase) for another 32 days. The control animals were still fed uncontaminated food. Prior to the start of the experiment (day 0) and after 2, 4, 6, 8, 16, 32 and 64 days (uptake phase) and 66, 68, 80 and 96 days (elimination phase), four individuals (two males and two females) from each temperature group were sacrificed at random. On days 16, 32 and 64 at 10 and 15°C, as well as on days 16 and 32 at 20°C, a few additional beetles were sampled for other purposes but were also analyzed for Ni concentrations and were thus included in this study. The control beetles were occasionally monitored to check for background Ni levels in their body. Before storing in the freezer, each beetle was kept in an empty box for 24 h to empty the gut content and weighed.

Experiment II: Effects of temperature on Ni toxicokinetics in larvae

One-day-old larvae of unknown sex were exposed to 300 mg Ni kg^{-1} food during 16-day uptake experiments at five different temperatures: 10, 15, 20, 22.5 and 25°C. Larvae were sampled after 1, 2, 4, 6, 8, 10, 14, 16 days of exposure. After 16 days, they were transferred to uncontaminated food for the next 8 days, and internal body concentrations were measured at days: 17, 18, 20, 22 and 24. Six larvae were sampled for chemical analysis on each sampling day. Before storing in the freezer (-20°C), each larvae was kept for 24 h in a punctured Eppendorf tube, wetted with distilled water to remove gut content and weighed to the nearest 0.01 mg (Sartorius, Germany). Larvae kept in the same soil but not exposed to Ni were occasionally monitored as a check for

'normal' behaviour and for background Ni levels in their body. Growth of the larvae was assessed on the basis of dry body mass of larvae sacrificed at each sampling time; the specific character of kinetics experiment did not tracking of the growth of the same larvae over the experiment; different larvae were weighed at each sampling time.

Experiment III: Effects of chlorpyrifos on Ni toxicokinetics in adult beetles

In another 96-day long experiment, adult beetles were exposed to Ni-contaminated food for 64 days (uptake phase) and afterwards were transferred to uncontaminated food (elimination phase). The sampling days were the same as in Experiment I, but this time four males were sampled each time. The following treatments were applied: control (Ni-0/CPF-0: 0 mg Ni kg^{-1} and 0 mg CHP kg^{-1}), acetone control (Ni-0/A: 0 mg Ni kg^{-1} and acetone), nickel (Ni-2500/CPF-0: 2500 mg Ni kg^{-1} and 0 mg CHP kg^{-1}), mix low (Ni-2500/CPF-10: 2500 mg Ni kg^{-1} plus 10 mg CHP kg^{-1}) and mix high (Ni-2500/CPF-30: 2500 mg Ni kg^{-1} plus 30 mg CHP kg^{-1}). Each treatment involved at least 55 beetles at the beginning of the experiment. Control beetles were monitored simultaneously to check for background Ni levels in their bodies. The sampled beetles were starved for 48 h, then weighed and stored at -20°C for Ni analysis.

Chemical analyses

After completion the experiment, the frozen adult beetles and food (three or four samples per treatment for each of the two or three batches) were dried at 105°C for 24 h and weighed to the nearest 0.001 g (WPA 180/k Radwag, Radom, Poland) before digesting in 1.5 ml boiling concentrated HNO_3 (Suprapur HNO_3, Merck) and then diluting to 5 ml with deionised water. Larvae were weighed to the nearest 0.01 mg (Sartorius, Germany), washed in deionised water to remove all remains of food from their body surface, desiccated on filter paper and then digested in 100 µl boiling HNO_3 and resuspended to 0.5 ml with deionised H_2O. Nickel concentrations in the beetles were analyzed with a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800; detection limit: 0.68 µg L^{-1}), and in food with flame AAS (detection limit: 0.13 mg L^{-1}). Three blanks and three samples of reference material (dogfish muscle DORM-2; National Research Council, Ottawa, ON, Canada) were run

with each batch to check the analytical precision of each method. The chemical analyses were accurate, as indicated by the results for the reference material: the certified value was 19.4 ± 3.1 mg kg^{-1}, and the measured concentrations were 20.7 ± 3.4 mg kg^{-1} (Experiment I), 15.0 ± 3.8 mg kg^{-1} (Experiment II) and 18.9 ± 2.8 mg kg^{-1} (Experiment III) for the graphite furnace AAS (beetles) and 24.3 ± 1.8 mg kg^{-1} for the flame AAS (food). The results were not corrected for recovery. Nickel concentration in beetles and in food was expressed as mg kg^{-1} dry mass. Because the food was prepared two or three times during the experiment, samples of each batch were mineralized and analysed.

Chlorpyrifos in food was analyzed by the Institute of Industrial Organic Chemistry, Pszczyna Branch, Poland, and was recovered via extraction twice with ethyl acetate and infiltration through sodium sulfate. After solvent evaporation the samples were dried and then re-dissolved in acetone for CPF estimation by gas chromatography (Agilent 6890N) with an electron capture detector (GC-ECD). CPF extraction efficiency was high (ca. 100%), and the detection limit of the method was 0.1 mg kg^{-1} food.

Statistical analysis

If mortality during the experiments was high enough, the data were assessed by survival analysis and the survival curves were compared between treatments with a log-rank test (Mentel, 1966). All beetles used in the experiments were included in survival analyses, with survival data censored for the beetles sampled for the kinetics study and/or larvae that escaped before the end of the experiment.

To check whether adult beetles lost no body mass over the experiment, the body mass change (BMC) index was calculated according to the following equation:

$$BMC = \frac{(M_{t_n} - M_{t_0})}{M_{t_0}}$$

where: M_{t_n} = mass of beetles at sampling day n (g)

M_{t_0} = mass of beetles at the beginning of the experiment (g)

The body mass and BMC index distributions were checked for normality with Shapiro-Wilk's W test. Because most of the data were not normally distributed, they were log-transformed and this satisfied the criterion in all cases. Differences in the initial mass of beetles assigned to different temperatures (Experiment I) or treatments (Experiment III) were analysed by multi-factor ANOVA with temperature and sex (Experiment I) or treatment (Experiment III) as factors. The BMC indices were compared between sampling days and temperatures (Experiment I) or treatments (Experiment III) with multi-factor ANOVA, and for each significant factor a post hoc LSD test was run to separate means. If a test conducted for all temperatures or treatments detected significant effect at $p<0.05$, BMC indices at each sampling day were compared with those at the beginning of the experiment (day 0) for each temperature (Experiment I) or treatment (Experiment III) separately.

Growth rate was ignoredm for adult beetles because maximum body size was already reached. The larval specific growth rate γ (day^{-1}) was determined based on dry body mass by fitting the Von Bertalanffy curve (Crommentuijn et al., 1997), which gave a reasonably good description during this life stage for each temperature tested:

$$M_{(t)} = \{M_{\infty}^{1/3} + (M_{\infty}^{1/3} - M_0^{1/3})\exp(-\gamma t)\}^3$$

where: $M_{(t)}$ = body mass at day t (mg dry mass)

M_{∞} = final body mass (mg dry mass)

M_0 = initial body mass (mg dry mass)

γ = specific growth rate (day^{-1})

t = time (days)

Differences between the estimated Von Bertalanffy growth parameters for each temperature were tested by comparing asymptotic 95% confidence intervals around the

estimated parameters; if the confidence intervals did not overlap, the difference between temperatures was considered statistically significant ($p<0.05$).

Two-phase and three-phase one-compartment models

The kinetics data were analysed with a classic one-compartment toxicokinetics model with a breakpoint set to the day when animals switched to decontamination (last day of uptake phase) (Atkins, 1969). The following equations were used to estimate assimilation rate (k_a) and decontamination rate (k_e):

For $t \leq tc$ (uptake phase):

$$C_t = C_0 + C_{\exp}\frac{k_a}{k_e}(1-e^{-k_e t})$$

For $t > tc$: (elimination phase):

$$C_t = C_0 + C_{\exp}\left(\frac{k_a}{k_e}(1-e^{-k_e t}) + \frac{k_a}{k_e}(1-e^{-k_e (t-t_c)})\right)$$

where: C_t = internal Ni concentration in the beetle body at time t (mg kg^{-1})

C_0 = background internal Ni concentration at t=0 (mg kg^{-1})

C_{exp} = exposure concentration in food (mg kg^{-1})

k_a = assimilation rate constant - fraction of Ni concentration in food that is assimilated per unit time (day^{-1}); the constant k_a represents the initial slope of the uptake phase (Van Straalen et al., 2005).

k_e = elimination rate constant (day^{-1})

t = exposure time (days)

t_c = time at which the beetles were transferred to uncontaminated food (days)

e = base of the natural logarithm

There are actually two ways of expressing and analysing toxicokinetics with these models. Either C_{exp} is included in the model, and then k_a is expressed as above, or the

model omits C_{exp}, and then k_a is expressed in mass of a chemical assimilated per body mass in time t (mg g^{-1} day^{-1}) (e.g., Janssen et al., 1991). Mathematically this make no difference, as one k_a can be recalculated to another simply by multiplying or dividing it, as appropriate, by the metal concentration in food.

It was observed an initial fast increase in nickel concentration (high assimilation, elimination non-existent or very slow), followed by a decrease in body concentration leading to some equilibrium concentration (k_a balanced by k_e either when metal assimilation is significantly decreased, efficient elimination mechanisms have been engaged, or both phenomena act in unison), eventually followed by a further decrease to the initial concentration when the beetles were transferred to uncontaminated medium and the physiological mechanisms were potent enough to purge the body completely of the contaminant. In view of this, it was concluded that the classic model neglects an important physiological mechanism and that a somewhat more elaborate toxicokinetic model should be used to describe the behaviour of nickel in the beetles. The modified model should allow for (1) an early switching point (D), until which animals assimilate the metal at a high rate and cannot efficiently excrete superfluous amounts of it and (2) at least two different assimilation constants, k_{a1} and k_{a2}, and/or elimination constants, k_{e1} and k_{e2}, describing the different assimilation and/or elimination efficiencies before and after the switching point. However, it was not possible to make estimates so many parameters from the data obtained in this study; the additional parameters in the formula made the estimates nonsignificant due to the low number of degrees of freedom. To deal with this difficulty only a modification of the classic model was made: instead of a fixed switching point (model breakpoint) set to the last day of the uptake phase (t_c), a breakpoint day D was estimated from the best-fit model. Such a model assumes that an animal stops assimilating toxic metal as soon as a certain internal concentration is reached, even if it is still exposed to metal-contaminated food. Both two-phase models (i.e., classic and estimated breakpoint) consider an animal as a homogenous compartment with a constant assimilation rate and a single elimination rate. Assimilation is assumed to be zero during the

decontamination phase ($t>t_c$ or $t>D$), and the body burden in the decontamination period decreases until the pre-exposure concentration C_0 is reached.

Additionally, because the data indicated that probably a three-phase model would really be probably best (there was a fairly clear mismatch in the intermediate phase between the estimated breakpoint and the day the animals were transferred to clean food), in Experiment III the data from Ni exposed-beetles were used to test a model with estimated breakpoint D, a single assimilation rate constant (k_a), two elimination rate constants (low during the initial quick concentration increase (k_{e1}) and another one for the subsequent equilibrium concentration and elimination phases (k_{e2})), asymptotic equilibrium concentration (A), and the asymptotic final concentration after decontamination (C_f). Equilibrium concentration A was introduced to the model to allow for the observed tendency to achieve a stable concentration during the uptake phase, lower than the peak concentration reached during the first days of the exposure. The asymptotic final concentration C_f allows for reaching a final concentration different from the initial concentrations, that is, for incomplete depuration of the assimilated metal. Theoretically, with more densely sampled animals the model could also be tested for separate assimilation rates, but the available data did not allow that. Moreover, at this point it does not really matter whether the decreased metal accumulation after the switching point (D) resulted from decreased metal assimilation or increased elimination (as we assumed here), or both. To sort out these two mechanisms, specifically designed studies would be necessary. The following equations for the three-phase model were used (Laskowski et al., *submitted*):

For $t \leq D$ (uptake phase):

$$C_t = C_0 + C_{\exp}\frac{k_a}{k_{e1}}(1 - e^{-k_{e1}t})$$

For $t > D \leq tc$ (uptake phase):

$$C_t = A + \left[\left(C_0 + C_{\exp} \frac{k_a}{k_{e1}} (1 - e^{-k_{e1} D}) \right) e^{-k_{e2}(t-D)} \right]$$

For $t > tc$: (elimination phase):

$$C_t = C_f + A \times e^{-k_{e2}(t-t_c)}$$

The model parameters were estimated by fitting the equation for all phases simultaneously using the Marquardt method. The Marquardt method was preferred for its robustness and consistency of results irrespectively of initial conditions (initial parameter values). All parameters were checked for significance using asymptotic 95% confidence intervals. The asymptotic intervals around estimated parameters were also used to compare treatments.

Because for some days the variance of Ni concentration was higher than for others, we ascribed more importance to the values with less scatter. When variance differs vastly between sampling dates, as was the case in this study, theoretically a weighted regression should result in more robust solutions. In the next step, therefore, a weighted regression models with weights inversely proportional to the variance of concentration at each sampling day were tested. Because weighted regression, like other least squares methods, is sensitive to the effects of outliers, the data points with absolute values of studentized residuals greater than 3.5 were excluded from regression analysis. The combined data for all temperatures or treatments were not cleansed of possible outliers, but the influence of extreme data points was weakened by using geometric mean concentrations. The models were compared according to their R^2 values adjusted for degrees of freedom (R^2_{adj}). To show how much of the total variance of data is explained by a particular model, we used raw data and the respective R^2 values. The models and R^2 values for geometric means described how well the models depict the general temporal toxicokinetic pattern.

Each individual was treated as an independent replicate. In Experiment I the data for both sexes were pooled together to increase the power of the analyses. Sex ratios were approximately equal for temperatures.

Two-way ANOVA with day and temperature (Experiment I and II) or treatment (Experiment III) as explanatory factors was used to determine the ability of beetles to depurate down to initial concentrations, by comparison Ni concentrations at days 0 and 96 (Experiments I and III) or 0 and 24 (Experiment II). If temperature or treatment were significant variables in the model ($p<0.05$), the differences between days were analysed separately for each of them, with Bonferroni correction for multiple comparison.

All analyses employed Statgraphics Centurion XV (Statpoint Inc.; www.statgraphics.com).

Results

The actual Ni concentrations in the food were in good accordance with the nominal ones in all experiments (Table 5.1). Nickel concentrations in the food from the Ni-only exposures did not significantly different ($p=0.8$) from those in which Ni was combined with CPF was used (Experiment III).

Experiment I: Effects of temperature on Ni toxicokinetics in adult beetles

The mortality of beetles in Experiment I did not exceed 10%. The initial body mass of females (0.0590 g) was significantly higher than of males (0.0537 g) ($p<0.0001$), with no significant differences between the temperatures to which they were assigned ($p=0.9$). Both temperature and sampling day significantly influenced the BMC index ($p<0.0001$), but with no clear time-dependent pattern at any temperature. After one week of acclimation (day 0) the average BMC index was significantly lower at 25°C than at 10, 15 and 22.5°C, and at 20°C it was significantly lower than at 10°C ($p=0.01$). Only the beetles at 25°C lost mass during acclimation, and those at 20°C put on less weight than at other temperatures. Although there were significant differences between days of exposure at all temperatures but 15°C ($p\leq0.03$), body mass loss versus

day 0 was significant only in two cases: at 10 and 22.5°C at day 4. Those differences appeared nonsignificant if Bonferroni-corrected for multiple comparisons. This means that the temperature effect during acclimation was an initial response which disappeared once the beetles were fully acclimated and had been exposed to Ni. There was no significant relationship between body mass and body Ni concentration during the uptake phase at any temperature except 25°C, for which there was a weak (r=0.5) but significant (p=0.005) positive correlation.

Table 5.1. Actual nickel concentrations in food used for the kinetics experiments with *Pterostichus oblongopunctatus* (mean ± SD). Sample sizes (number of food batches analyzed) are given in brackets.

		Ni concentration mg kg^{-1}		CPF concentration mg kg^{-1}	
Experiment	Series	Nominal	Actual	Nominal	Actual*
I	I	0	7.6 ± 1.3 (4)	–	–
		2500	2760 ± 157 (4)	–	–
	II	0	2.6 ± 1.1 (4)	–	–
		2500	2526 ± 81 (4)	–	–
II	I	0	10.6 ± 4.2 (3)	–	–
		300	376 ± 31 (4)		–
III	I	0	7.4 ± 1.2 (3)	0	0
		0	4.6 ± 0.5 (3)	Acetone	0
		0	7.4 ± 0.2 (3)	10	12.2
		0	5.8 ± 0.6 (3)	30	30.4
		2500	2525 ± 76 (3)	0	0
		2500	2477 ± 135 (3)	10	13.7
		2500	2542 ± 129 (3)	30	34.0
	II	0	3.3 ± 0.7 (3)	0	0
	III	0	7.2 ± 1.4 (3)	0	–

* analyzed in Institute of Industrial Organic Chemistry, Branch Pszczyna

Among the 294 values, 16 outliers (ca. 5%) were excluded from the nonlinear regression analysis in the Experiment I.

An unexpected pattern of Ni kinetics was found at all temperatures: the metal concentration increased rapidly to maximum after a few days of exposure and then slowly but consistently decreased even if the animals were still fed Ni-contaminated food. This, along with high variability of internal Ni concentrations observed especially in the uptake phase was why the classic one-compartment model with a fixed breakpoint day appeared inadequate and in most cases either could not be fitted to the data at all or else yielded nonsignificant estimated parameters (Table 5.2). The model with an estimated breakpoint fitted the data better; the estimated parameters were significant at all temperatures but 15°C (asymptotic 95% confidence intervals for k_e covered 0) and explained 31 - 47% or (weighted regression) 57 - 74% of the total variability of Ni body concentrations (Table 5.3, Fig. 5.1).

Because no differences in kinetics parameters between temperatures (10, 15, 20, 22.5 and 25°C) were found in either model, the results from all temperatures were pooled and analysed together. The weighted estimated breakpoint model for combined data gave the assimilation rate constant k_a =0.020 day^{-1} which equals an accumulation rate of about 50 mg Ni kg^{-1} day^{-1}, and elimination rate constant k_e = 0.014 day^{-1}. The estimated breakpoint indicated that the beetles started to decontaminate after ca. 2.5 days after the start of exposure to contaminated food. When the model was applied to geometric means, the breakpoint day was ca. 5 days and as much as 91.5% of the temporal variability of Ni body concentrations was explained (Fig. 5.2). Estimates from both normal and weighted regression models are summarised in Tables 5.2 and 5.3, respectively.

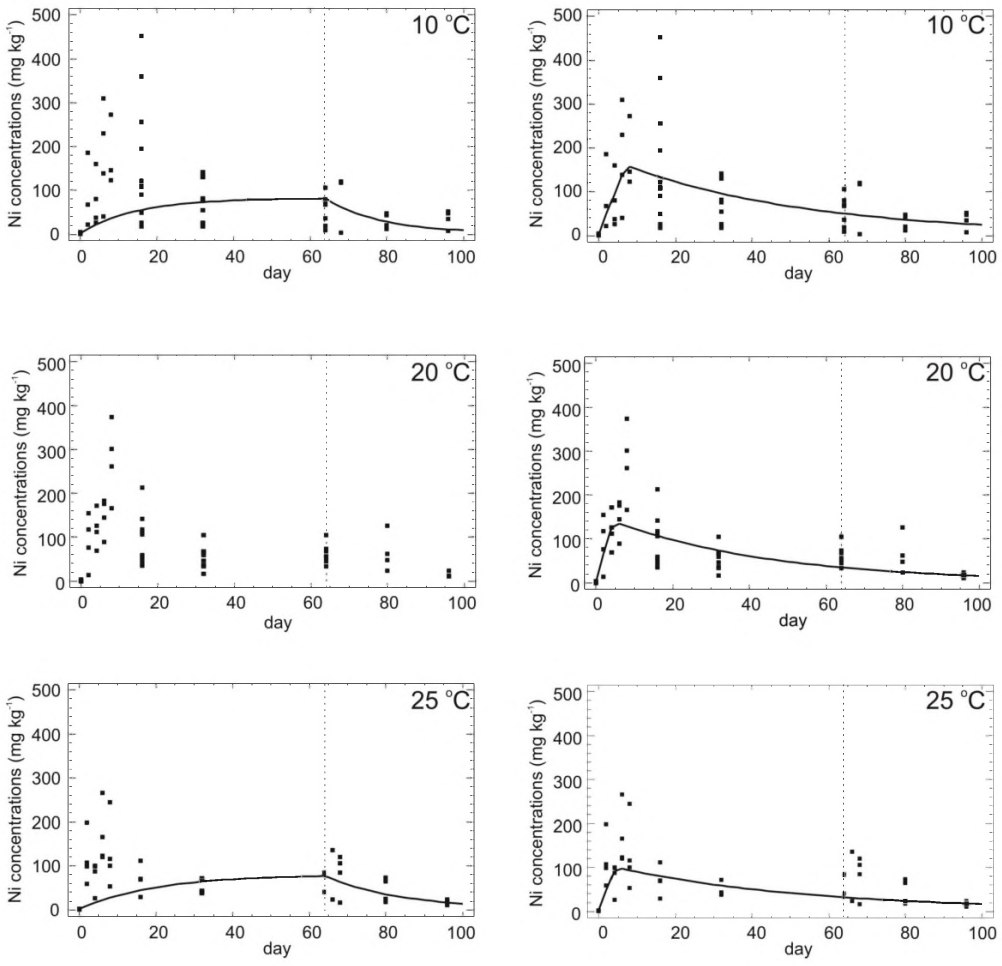

Fig. 5.1. Nickel kinetics in adult *Pterostichus oblongopunctatus* exposed at 10°C (top row), 20°C (middle row) and 25°C (bottom row); solid line indicates the fitted classic one compartment model (left-hand column) or estimated breakpoint model (right-hand column); vertical broken line indicates the day of transfer to uncontaminated food. For parameters estimates see Table 5.3.

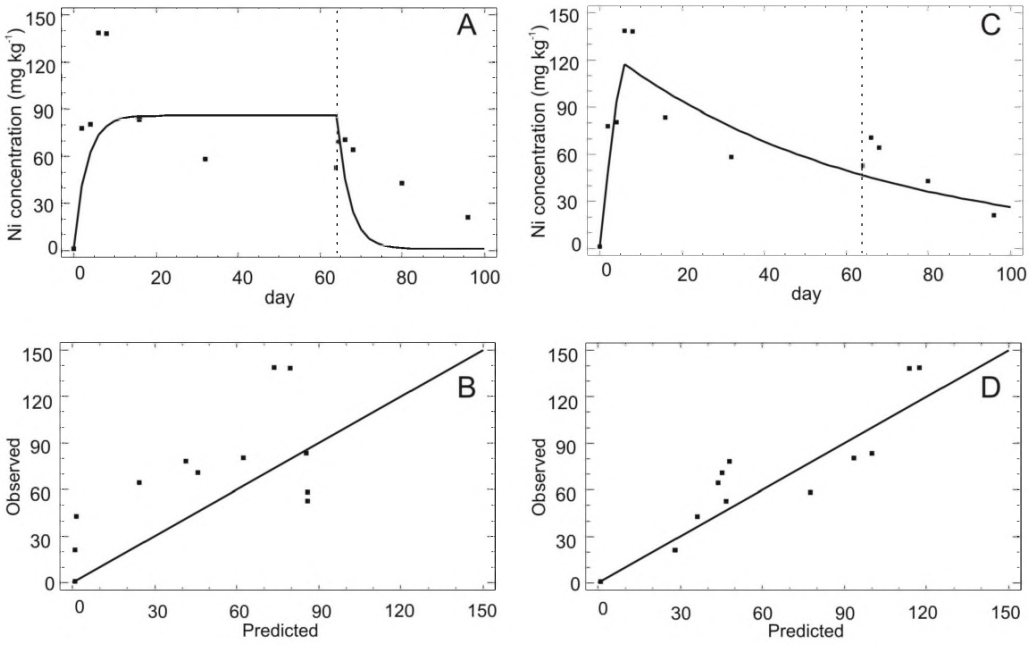

Fig. 5.2. Nickel kinetics in adult *Pteristichus oblongopunctatus* described by the classic one-compartment model (A) or estimated breakpoint model (C) and observed Ni concentrations versus predicted by the fitted classic (B) and breakpoint (D) models. The models were fitted to geometric mean concentrations for combined data from all temperatures; vertical broken line indicates the day of transfer to uncontaminated food. For parameters estimates see Table 5.3.

At the end of the Experiment I the Ni levels in the beetles were much higher than at the beginning of the experiment at all temperatures ($p \leq 0.03$; Table 5.3), but the differences between days 0 and 96 at 15 and 22.5°C appeared nonsignificant when Bonferroni-corrected for multiple comparisons. The Ni concentrations in control beetles at 16, 32, 64 and 96 days were below the level at the beginning of the experiment (Table 5.4).

Table 5.2. Parameters estimated and asymptotic 95% confidence intervals for the classic one-compartment model and the model with estimated breakpoint to the data obtained for Ni toxicokinetics in adults *Pterostichus oblongopunctatus* at different temperatures; parameters estimated on raw data and geometric means (geomean); NS – parameter nonsignificant, R^2_{adj} – *R*-squared adjusted for d.f., C_0, C_{96} – Ni concentrations (mean ± SD) at the beginning (day 0) and end of the experiment (day 96), respectively, — means that model could not be fitted to the data

			Estimated parameters						
			Classic one-compartment model			Model with estimated breakpoint			
Temperature °C	C_0 mg kg^{-1}	C_{96} Mg kg^{-1}	k_a day^{-1}	k_e day^{-1}	R^2 R^2_{adj} %	k_a day^{-1}	k_e day^{-1}	D days	R^2 R^2_{adj} %
10	2.32±2.06 (4)	36.1±20.3 (4)	0.022 NS	0.46 NS	13.2 11.7	0.011 0.007–0.016	0.024 0.009–0.039	7.1 3.84–10.3	33.2 31.0
15	3.84±1.86 (3)	36.1±23.3 (4)	—	—	—	0.026 0.016–0.037	0.015 0.006–0.023	2.1 1.14–2.97	33.5 31.3
20	0.93±0.81 (14)	14.3±6.6 (4)	0.061 NS	1.59 NS	31.6 30.5	0.018 0.007–0.030	0.025 0.012–0.039	3.6 1.33–5.91	48.5 46.8
22.5	1.78±1.65 (2)	31.4±26.9 (4)	0.009 0.003–0.016	0.22 0.066–0.37	0.0 0.0	0.012 0.002–0.022	0.005 NS	3.2 0.43–5.91	15.7 11.7
25	1.68±0.71 (4)	17.4±5.2 (4)	0.021 0.002–0.04	0.47 0.022–0.92	11.2 9.2	0.023 0.013–0.033	0.012 0.004–0.021	2.1 1.03–3.07	37.1 34.1
All Temperatures	1.63±1.47 (27)	27.1±19.3 (20)	0.024 0.011–0.038	0.60 0.25–0.95	9.8 9.4	0.020 0.014–0.025	0.013 0.010–0.017	2.7 1.95–3.46	32.1 31.5
All Temperatures (geomean)	0.93 (27)	21.3 (20)	0.016 NS	0.39 NS	24.5 16.9	0.010 0.006–0.004	0.015 0.008–0.023	5.4 2.91–7.96	80,3 75.9

Table 5.3. Parameters estimated and asymptotic 95% confidence intervals for the weighted classic one-compartment model and the weighted model with estimated breakpoint to the data obtained for Ni toxicokinetics in adults *Pterostichus oblongopunctatus* at different temperatures; parameters estimated on raw data and geometric means (geomean); NS – parameter nonsignificant, R^2_{adj} – *R*-squared adjusted for d.f., C_0, C_{96} – Ni concentrations (mean ± SD) at the beginning (day 0) and end of the experiment (day 96), respectively, — means that model could not be fitted to the data

			Estimated parameters						
			Classic one-compartment model			Model with estimated breakpoint			
Temperature °C	C_0 mg kg^{-1}	C_{96} mg kg^{-1}	k_a day^{-1}	k_e day^{-1}	R^2 R^2_{adj} %	k_a day^{-1}	k_e day^{-1}	D days	R^2 R^2_{adj} %
10	2.32±2.06 (4)	36.1±20.3 (4)	0.0023 0.0006–0.004	0.069 0.025–0.114	45.5 43.1	0.010 0.0047–0.015	0.021 0.0135–0.028	7.0 2.99–11.1	67.0 65.8
15	3.84±1.86 (3)	36.1±23.3 (4)	—	—	—	0.026 0.0035–0.049	0.014 0.0065–0.022	2.1 0.26–3.94	73.3 72.8
20	0.93±0.81 (14)	14.3±6.60 (4)	—	—	—	0.013 0.0068–0.019	0.025 0.0167–0.032	4.5 2.11–6.91	58.5 57.2
22.5	1.78±1.65 (2)	31.4±26.9 (4)	0.0014 0.0009–0.002	0.036 0.016–0.056	74.1 73.5	0.012 0.0051–0.019	0.0045 NS	2.4 0.89–3.95	82.1 81.3
25	1.68±0.71 (4)	17.4±5.16 (4)	0.0016 0.0009–0.002	0.050 0.034–0.066	56.9 57.2	0.0093 0.0053–0.013	0.0196 0.014–0.0260	4.4 2.15–6.68	75.6 73.8
All Temperatures	1.63±1.47 (27)	27.1±19.3 (20)	0.018 0.0053–0.032	0.546 0.143–0.095	53.2 53.0	0.020 0.014 –0.026	0.014 0.0101–0.017	2.4 1.66–3.23	68.5 68.2
All Temperatures (geomean)	0.93 (27)	21.3 (20)	0.011 NS	0.32 NS	69,7 66.6	0.0095 0.0046–0.015	0.016 0.0088–0.023	5.2 2.06–8.26	93.1 91.5

Table 5.4. Nickel concentrations [mg kg^{-1}] in adults *Pterostichus oblongopunctatus* from control treatments in Experiment I and Experiment III (mean ± SD). Sample sizes (number of individuals analyzed) are given in brackets.

Experiment			Day 0	16	32	64	96
I	Temperature [°C]	10	2.32±2.06 (4)	0.80±0.64 (5)	1.8±1.60 (4)	0.36±0.30 (2)	1.13 (1)
		15	3.84±1.86 (3)	0.87±0.61 (3)	2.02±1.72 (9)	0.77±0.76 (2)	0.35±1.2 (2)
		20	0.93±0.81 (14)	0.87±0.53 (5)	0.79±1.06 (4)	0.31±0.17 (2)	0.41 (1)
III	CHP [mg kg^{-1}]	0	0.85±0.53 (21)	0.79±0.53 (7)	1.58±2.45 (7)	0.46±0.27 (7)	1.46±1.1 (9)

Experiment II: Effects of temperature on Ni toxicokinetics in larvae

Larvae mortality was ca. 30%, so the number of sampled larvae was reduced from 6 to 3 at days 10, 14, 17, 18, 20, 22 and 24 in Experiment II.

The average dry body mass of larvae at the beginning of Experiment II (one-day-old larvae) was 0.47 mg. There were significant differences in larval growth rate (on a dry mass basis) between temperatures: the larval specific growth rate γ and final mass M_∞ at 10°C differed significantly from those at higher temperatures (Table 5.5).

Among 310 values, 44 outliers (ca. 14%) were excluded from Experiment II.

As in the experiment on adults (Experiment I), Ni concentrations in larvae peaked after 2-3 days. Here again, the classic one-compartment toxicokinetic model with a fixed breakpoint day did not fit the experimental data at all and the parameter values could not be estimated (Table 5.6). The alternative model allowing for a non-fixed breakpoint day gave a better description of the data and indicated significant differences in the estimated parameters between temperatures. The assimilation rate constant k_a was lowest at two marginal temperatures (10 and 25°C) and differed significantly from that under 15°C, at which k_a was highest. Also, significant were the

differences in k_a between 10 and 20°C, 15 and 22.5°C, and 20 and 25°C. Larvae started to decontaminate significantly faster (about a day after the start of exposure to contaminated food) in the 15°C treatment than at 10 and 22.5°C (after ca. 3 days) (Table 5.6, Fig. 5.3). Using weighted regression considerably improved the model fit only for 10 and 15°C (Table 5.7).

Table 5.5. Effect of temperatures on body growth of *Pterostichus oblongopunctatus* larvae. Shown are the estimated specific growth rates (γ in day^{-1}) and final dry mass (M_∞ in mg) and asymptotic 95% confidence intervals. R^2_{adj} – R-squared adjusted for d.f.

	Estimated parameters		
Temperature °C	M_∞ Mg	γ day^{-1}	R^2_{adj} %
10	1.3 **B** (1.18–1.46)	0.33 **A** (0.14–0.52)	24.5
15	8.9 **A** (1.51–16.3)	0.04 **B** 0.011–0.072)	78.4
20	10.9 **A** (2.93–18.8)	0.04 **B** (0.016–0.074)	79.6
22	13.8 **A** (1.56–26.0)	0.04 **B** (0.009–0.076)	66.9
25	6.6 **A** (1.96–11.1)	0.06 **B** (0.017–0.11)	65.4

* the same letter means no significant differences between treatments

Nickel concentrations detected in larvae sampled at the end of the experiment were highest at both marginal temperatures (ca. 10 mg kg^{-1} at 10°C and 17 mg kg^{-1} at 25°C), and below 1.5 mg kg^{-1} at all other temperatures. Differences versus the initial concentration (8.2 mg kg^{-1}) were significant only at 15 and 22.5°C (p=0.01 after Bonferroni correction for multiple comparisons). The Ni concentrations in control beetles remained between ca. 6.0 and 25 mg kg^{-1} throughout the experiment (Table 5.8).

Table 5.6. Parameters estimated and asymptotic 95% confidence intervals for the classic one-compartment model and the model with estimated breakpoint to the data obtained for Ni toxicokinetics in larvae *Pterostichus oblongopunctatus* at different temperatures; parameters estimated on raw data; NS – parameter nonsignificant, R^2_{adj} – *R*-squared adjusted for d.f., C_{24} – Ni concentrations (mean ± SD) at the end of the experiment (day 24), — means that model could not be fitted to the data, NA – not analyzed

		Estimated parameters						
		Classic one-compartment model			Model with estimated breakpoint			
Temperature °C	C_{24} mg kg^{-1}	k_a day^{-1}	k_e day^{-1}	R^2 R^2_{adj} %	k_a day^{-1}	k_e day^{-1}	D days	R^2 R^2_{adj} %
10	10.4±7.92 (5)	0.15 0.033–0.3	1.11 0.19–2.0	34.0 33.0	0.077 **A** 0.046-0.107	0.064 0.0267-0.103	2.7 **B** 1.48–3.96	45.7 44.1
15	1.46±0.50 (3)	—	—	—	0.30 **B** 0.217–0.372	0.097 0.0481–0.146	1.0 **A** 0.62–1.38	63.4 62.3
20	0.87±0.50 (3)	0.78 NS	4.45 NS	46.4 45.6	0.22 **BC** 0.147–0.285	0.09 0.0481–0.130	1.3 **AB** 0.79–1.77	50.0 48.5
22.5	1.3±0.59 (5)	—	—	—	0.11 **A C** 0.068–0.148	0.14 0.0429–0.242	2.8 **B** 1.478–4.04	44.9 43.2
25	17.0±3.03 (2)	0.34 NS	3.4 NS	41.1 40.1	0.086 **A** 0.055–0.117	0.124 0.0297–0.219	2.2 **AB** 1.0–3.33	45.8 43.9
All Temperatures	5.53±7.07 (18)	—	—	—	NA	NA	NA	NA
All Temperatures (geomean)	2.53 (18)	—	—	—	NA	NA	NA	NA

* the same letter means no significant differences between treatment

Table 5.7. Parameters estimated and asymptotic 95% confidence intervals for the weighted classic one-compartment model and the weighted model with estimated breakpoint to the data obtained for Ni toxicokinetics in larvae *Pterostichus oblongopunctatus* at different temperatures; parameters estimated on raw data; NS – parameter nonsignificant, R^2_{adj} – R-squared adjusted for d.f., C_{24} – Ni concentrations (mean ± SD) at the end of the experiment (day 24)

	Estimated parameters							
	Classic one-compartment model				Model with estimated breakpoint			
Temperature °C	C_{24} mg kg^{-1}	k_a day^{-1}	k_e day^{-1}	R^2 R^2_{adj} %	k_a day^{-1}	k_e day^{-1}	D days	R^2 R^2_{adj} %
10	10.4±7.92 (5)	0.019 0.016–0.002	0.19 0.158–0.221	87.7 87.6	0.126 0.073–0.18	0.09 0.078–0.103	2.3 1.4–3.2	89.7 89.4
15	1.46±0.50 (3)	0.167 0.123–0.209	0.93 0.659–1.194	88.0 87.9	0.230 0.18–0.28	0.16 0.112–0.207	0.94 0.94–0.94	71.0 70.1

Table 5.8. Nickel concentrations [mg kg^{-1}] in *Pterostichus oblongopunctatus* larvae from control treatment in Experiment II (mean ± SD). Sample sizes (number of individuals analyzed) are given in brackets.

			Day			
Experiment			0	8	16	24
II	Temperature [°C]	10	8.3±5.60 (17)	25.9±28.5 (2)	11.4±6.4 (2)	23.02 (1)
		15		18.8±22.5 (2)	19.0±3.4 (2)	–
		20		28.0±16.3 (2)	5.9±0.81 (2)	9.7±4.9 (2)
		25		12.4±11.2 (2)	5.94 (1)	–

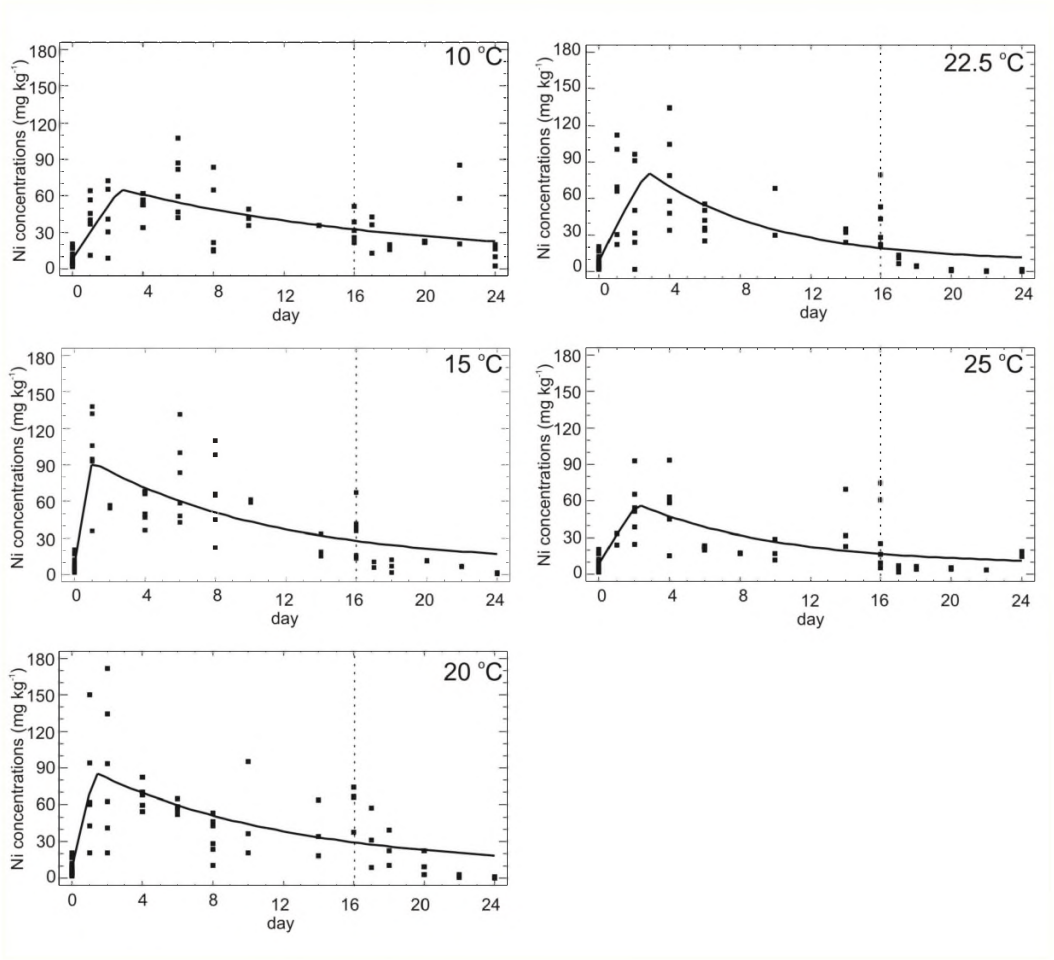

Fig. 5.3. Nickel kinetics in *Pteristichus oblongopunctatus* larvae at 10, 15, 20, 22.5 and 25 °C described by the estimated breakpoint model; vertical broken line indicates the day of transfer to uncontaminated food. For parameters estimates see Table 5.6.

Experiment III: Effects of chlorpyrifos on Ni toxicokinetics in adult beetles

Out of 276 beetles used at the start of the Experiment III, 47 died before the end of it. Almost no mortality occurred in treatments without CPF: 4 beetles died in Ni-0/CPF-0, one in Ni-0/A and 2 in Ni-2500/CPF-0. There was no significant effect of acetone on survival (p=0.1), so the data from Ni-0/CPF-0 and Ni-0/A were combined and used as a control. The survival curves for all exposure conditions differed significantly (p<0.0001), and in all treatments except Ni-2500/CPF-0 the beetles had significantly

higher mortality than the control. Mortality under Ni-2500/CPF-30 was higher than under Ni-2500/CPF-10 (Fig. 5.4).

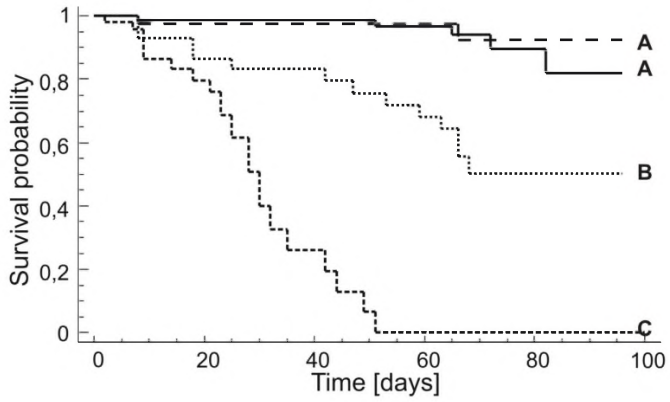

Fig. 5.4. Survival of *Pterostichus oblongopunctatus* exposed to various Ni and CPF concentrations in food (—— Ni-0/CPF-0, – – – – Ni-2500/CPF-0, ····· Ni-2500/CPF-10, – – – – – Ni-2500/CPF-30) in Experiment III. The same letter means no significant differences in lifetime between the treatments.

There were significant differences in initial body mass in Experiment III ($p=0.003$). The initial body mass of control beetles was significantly higher than in other treatments except Ni-2500/CPF-0, where it was also higher than in Ni-2500/CPF-10. Similarly to Experiment I, both treatment and sampling day influenced the BMC index ($p<0.0001$): it was significantly lower in Ni-2500/CPF-30 than in the other treatments, and lower in Ni-2500/CPF-10 than in the control (Ni-0/CPF-0). Significant differences between days of exposure were noted in both CPF treatments: there was body mass loss in versus day 0 at days 16, 80 and 96 in Ni-0/CPF-10, and gradual body mass loss (negative BMC index) in 2500/CPF-30 at all sampling days starting with day 6. All differences but one (day 96 in Ni-0/CPF-10) disappeared when Bonferroni correction.

Among the 132 values only 4 outliers (ca. 3%) were identified and excluded from nonlinear regression analysis the Experiment III.

Because it turned out that the beetles were very sensitive to a high level of CPF (30 mg kg^{-1} food) and no animals survived longer than 32 days of exposure, the data for Ni kinetics under high CPF are not complete. The data for Ni uptake and elimination kinetics in animals exposed to Ni alone and in combination with low level of CPF (10 mg kg^{-1} food) are complete and were used for comparison of kinetics patterns between treatments. With the procedures used it was not possible to fit any of the models with normal (i.e. unweighted) regression approach. Only weighted regression fit both models satisfactorily, and the breakpoint model proved better (R^2_{adj} = 54.1%, vs 32.7% for the classic model; Table 5.9, Fig. 5.5). Neither k_a nor k_e was affected by CPF when the estimated breakpoint model was analysed, and only a minor difference between treatments was found for the breakpoint day D. Despite those differences, the estimated breakpoint model fitted the combined raw data and the geometric means for both treatments. Analysis of combined raw data gave the following parameter values: k_a = 0.023, k_e = 0.031 and D = 1.23. Analysis of geometric means gave 0.032, 0.030 and 1,14 respectively (Table 5.9, Fig. 5.6).

The three-phase model used for beetles exposed exclusively to Ni (Ni-2500/CPF-0) improved the fit significantly (R^2_{adj} = 69.0%) versus both the classic and estimated breakpoint models and a clear change in Ni toxicokinetics in the course of the experiment was made particularly evident when the model was fitted to geometric means rather then to raw data (Table 5.10, Fig. 5.7). In this model the breakpoint D was estimated at 1.4 days, and after the breakpoint the Ni concentration levelled out at ca. 34 mg kg^{-1}. The assimilation rate k_a was 0.093 day^{-1}. The elimination rate in the first phase (k_{e1} = 0.11 day^{-1}) was about eight times lower than in the later two phases (k_{e2} = 0.89 day^{-1}). Using weighted regression did not substantially improved the model fit.

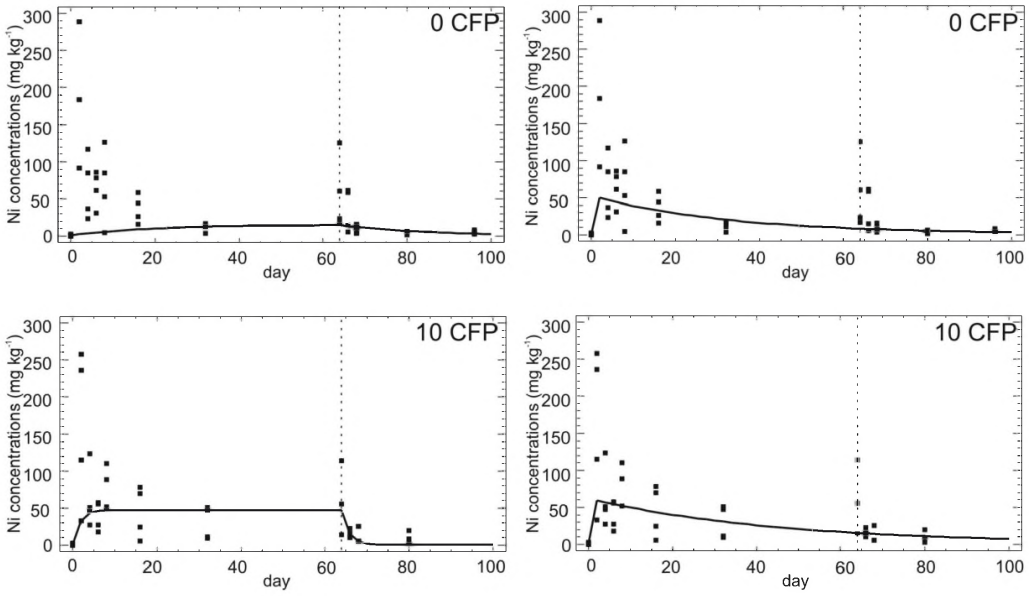

Fig. 5.5. Nickel kinetics in adult *Pterostichus oblongopunctatus* exposed to Ni-contaminated food without chlorpyrifos (upper row) and with 10 mg kg^{-1} chlorpyrifos (lower row); solid line indicates the fitted classic one-compartment model (left-hand column) or estimated breakpoint model (right-hand column); vertical broken line indicates the day of transfer to uncontaminated food. For parameters estimates see Table 5.9.

Table 5.9. Parameters estimated and asymptotic 95% confidence intervals for the weighted classic one-compartment model and the weighted model with estimated breakpoint to the data obtained for Ni toxicokinetics in *Pterostichus oblongopunctatus* under the influence of chlorpyrifos (CPF); parameters estimated on raw data and geometric means (geomean); NS – parameter nonsignificant, R^2_{adj} – R-squared adjusted for d.f., C_0, C_{96} – Ni concentrations (mean ± SD) at the beginning (day 0) and at the end of the experiment (day 96), respectively

			Estimated parameters						
			Classic one-compartment model			Model with estimated breakpoint			
CPF mg kg^{-1}	C_0 mg kg^{-1}	C_{96} mg kg^{-1}	k_a day^{-1}	k_e day^{-1}	R^2 R^2_{adj} %	k_a day^{-1}	k_e day^{-1}	D days	R^2 R^2_{adj} %
0	0.85±0.53 (21)	4.9±2.01 (8)	0.0003 **A** 0.00005–0.0005	0.05 **A** 0.025–0.08	33.7 32.7	0.011 0.0069–0.014	0.030 0.023–0.037	1.85 **A** 1.85–1.85	55.4 54.1
10	0.85±0.53 (21)	2.35 (1)	0.011 **B** 0.0045–0.017	0.59 **B** 0.34–0.84	55.8 55.0	0.012 0.0086–0.016	0.022 0.009–0.030	1.92 **B** 1.92–1.92	60.6 59.1
0 and 10	0.85±0.53 (21)	4.63±2.06 (9)	0.008 0.0031–0.012	0.45 0.21–0.69	40.6 40.1	0.023 0.018–0.028	0.031 0.026–0.352	1.23 1.23–1.23	60.4 59.6
0 and 10 (geomean)	0.71±0.53 (21)	4.24	0.006 NS	0.46 NS	55.9 51.5	0.032 0.013–0.027	0.030 0.022–0.037	1.14 1.14–1.14	88.6 86.1

* the same letter means no significant differences between treatments

Table 5.10. Parameters estimated and asymptotic 95% confidence intervals for the three-phase one-compartment model to the data obtained for *Pterostichus oblongopunctatus* exposed to food contaminated with Ni at 2500 mg kg^{-1} (Experiment III); parameters estimated on raw data and geometric means (geomenan); NS – parameter nonsignificant, R^2_{adj} – *R*-squared adjusted for d.f., C_0 – Ni concentrations (mean ± SD) at the beginning (day 0) of the experiment

	Estimated parameters							
	Three-stage model							
	C_0 mg kg^{-1}	k_a day^{-1}	k_{e1} day^{-1}	k_{e2} day^{-1}	D days	A mg kg^{-1}	C_f mg kg^{-1}	R^2 R^2_{adj} %
raw data	0.85±0.53 (21)	0.094 0.08–0.11	0.16 0.159–0.16	0.90 NS	1.5 1.45–1.5	44.7 NS	9.9 NS	71.3 69.0
geomean	0.71±0.53 (21)	0.093 0.074–0.112	0.11 0.11–0.12	0.89 NS	1.38 1.37–1.38	34.4 NS	8.2 NS	96.3 93.2

The Ni concentrations in beetles sampled at the end of the experiment was below 5 mg kg^{-1} in both treatments (Table 5.9), but because the Ni concentrations at the beginning of the experiment were very low, the differences between days 0 and 96 were significant (p<0.0001). The Ni concentrations in control beetles were below 1.6 mg kg^{-1} throughout the experiment (Table 5.4).

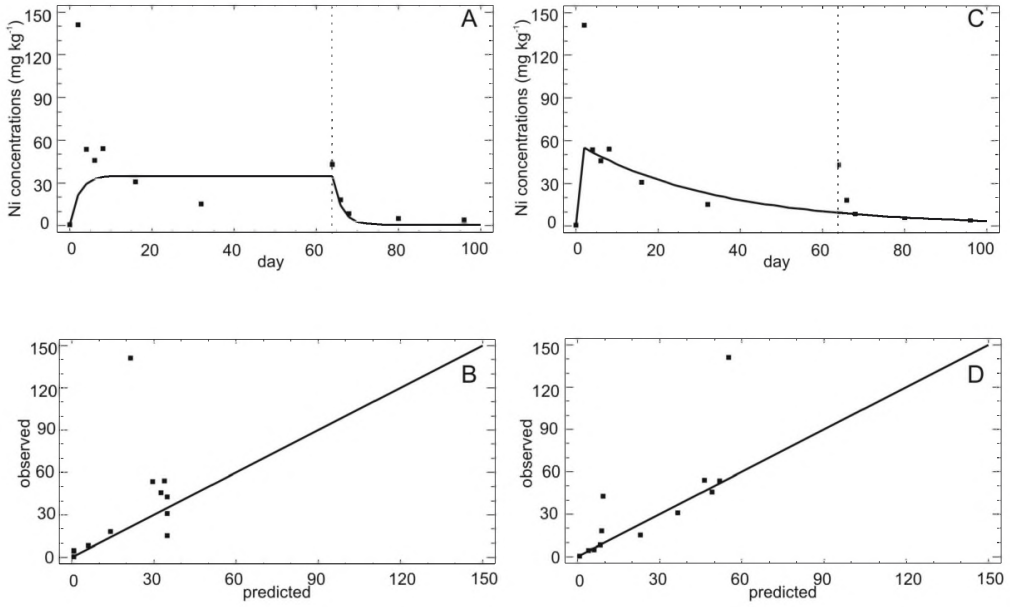

Fig. 5.6. Nickel kinetics in adult *Pteristichus oblongopunctatus* described by the classic one-compartment model (A) or estimated breakpoint model (C), and observed Ni concentrations versus values predicted by the fitted classic (B) and breakpoint (D) models. The models were fitted to geometric mean concentrations for combined data from treatment with and without chlorpyrifos; vertical broken line indicates the day of transfer to uncontaminated food. For parameters estimates see Table 5.9.

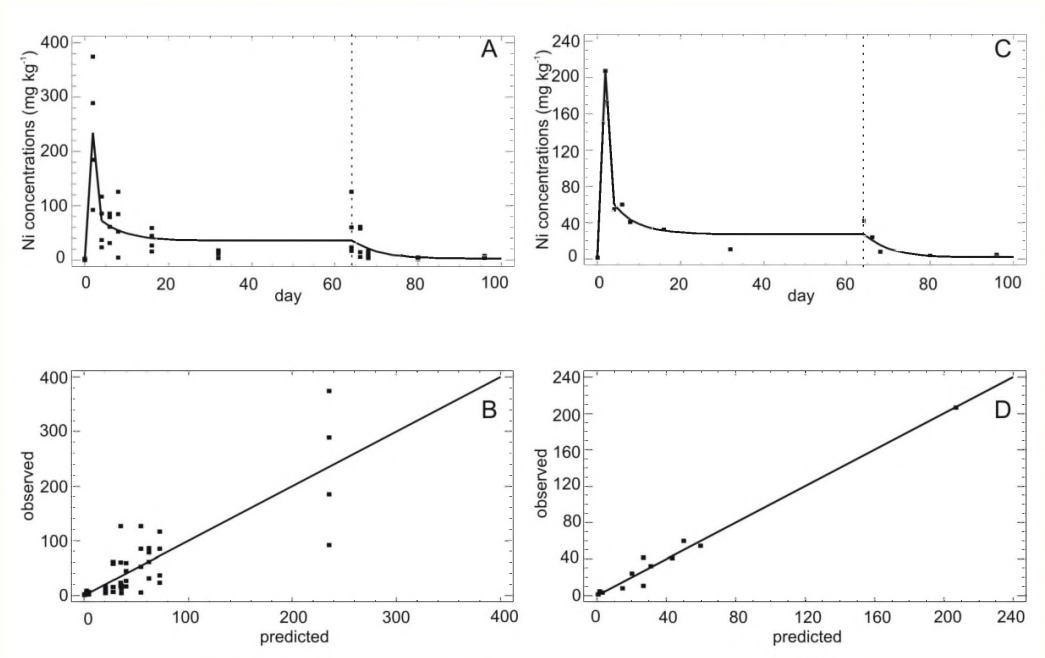

Fig. 5.7. Nickel kinetics in adult *Pteristichus oblongopunctatus* described by the three-phase model. The model was fitted to the raw data (A) and to geometric mean concentrations (C) for combined data from treatments with and without chlorpyrifos; vertical broken line indicates the day of transfer to uncontaminated food. For parameters estimates see Table 5.10.

Discussion

The most interesting finding of this study was that the beetles tended to switch to efficient elimination of Ni early in the uptake phase, irrespective of temperature or treatment with CPF: both adults and larvae started to decontaminate after only two to five days of exposure to the metal, on average. Such an unexpected pattern of Ni accumulation made it impossible in many cases to use the classic one-compartment model to analyse the data. In their study on Zn and Cd kinetics in the same species, Lagisz et al. (2005) also observed metal elimination already in the uptake phase. This did not allow them to fit one-compartment models to their data and compare F_1 generations of the beetles originating from polluted and references areas. Their results were especially unexpected, since uptake and elimination kinetics consistent with expectations of the classic one-compartment model were observed for the same metals

in the closely related carabid *Poecilus cupreus* (Kramarz, 1999a). One may conjecture that the differences observed between results for *P. oblongopunctatus* (Lagisz et al., 2005; this study) and for *P. cupreus* were due to species biology and/or the experimental setup, for example, the use of different medium to feed the beetles. In this study and in Lagisz et al. (2005) the beetles were offered artificial food (ground mealworms mixed with ground apple and $NiCl_2 \times 6H_2O$, chicken meat mixed with $CdCl_2 \times 2.5H_2O$ and/or $ZnCl_2$, respectively), whereas Kramarz (1999a) fed the beetles with housefly larvae reared on artificial medium. The bioavailability of metals may have differed between those media. On the other hand, although Janssen et al. (1991) fed the carabid *Notiophilus biguttatus* with natural food (collembolans *Orchesella cincta* raised on green algae contaminated with $CdSO_4$), they also found substantial deviations from the classic one-compartment model during the accumulation period (see original data shown in their Fig. 2D). They suggested that such deviations might be due to experimental errors not explained by the model. In the light of more recent data, however, such a decrease in metal level already during the uptake phase seems not so exceptional. Ni kinetics patterns similar to what was found in the present study, as well as high variance in internal Ni concentrations, have also been reported in the earthworm *Lumbricus rubellus* (Svendsen et al., personal comm.) and springtail *Folsomia candida* (Broerse and Van Gestel, personal comm.). Nauhauser et al. (1995) studied five metals (Cd, Cu, Ni, Pb and Zn) in the earthworm *Allolobophora tuberculata* Eisen; they found unexpected patterns of Ni uptake and elimination kinetics, Pb and Cu uptake kinetics, and Pb elimination kinetics. Specifically, the Ni, Pb and Cu concentrations increased rapidly in the uptake phase (worms transferred from uncontaminated to contaminated soil) to levels that might be expected in worms at steady-state kinetics, and than slowly but consistently decreased to the levels seen in them before they were transferred to contaminated soil. In the elimination studies (worms transferred from contaminated to uncontaminated soil) the Ni and Pb concentrations decreased initially but then followed an irregular pattern over the rest of the study (Nauhauser et al., 1995). In the centipede *Lithobius forficatus* the Cd level also increased dramatically at first and than steadily decreased even though the animals

were regularly fed Cd-contaminated *Chironomus* larvae (Descamps et al., 1996). Similarly, fast initial accumulation followed by rapid excretion already during the contamination phase was found in Cu-exposed earthworms *Eisenia fetida* (Spurgeon and Hopkin, 1999). Thus, for different metals and different species the same phenomenon has been observed: a fast increase in concentration very early in the uptake phase, followed by a decrease of metal concentration while animals were still being exposed to metal-contaminated medium, and then a further decrease - as might be expected from Atkins' (1969) classic model - when the animals were transferred to uncontaminated medium. This means that the unexpected Ni kinetics found in this study are neither species-specific nor related specifically to Ni.

The present studies, together with a review of the published papers, indicate that metal toxicokinetics cannot always be adequately described by the classic two-phase model with one accumulation constant and one elimination constant, and a fixed breakpoint. Possibly, the early-phase deviations from the classic model, showing concentrations substantially exceeding those predicted from it, are not simply "outliers" or analytical errors but rather indicators of an important physiological mechanism. It seems reasonable that an animal exposed to an extreme concentration of a metal needs some time to adjust its physiology to more effective metal regulation. This mechanism may be a biochemically mediated change in the physiology of metal regulation, or a simple outcome of the poisoning of gut epithelial cells and massive replacement of dead cells with new ones. Such a discharge of gut epithelium cells that have taken up metals has been implicated in the increase in metals excretion efficiency in metal-exposed invertebrates: the collembolan *Orchesella cincta* (L.) (Posthuma et al., 1992) and ground beetle *Pterostichus niger* (Lindqvist et al., 1995). Whatever the mechanism, it results in decreased metal assimilation rate or an increased metal elimination rate, or both working together.

This study showed that the classic two-phase toxicokinetic model is not adequate at least in the case of beetles exposed to Ni-contaminated food. Using a two-phase model with estimated breakpoint improved the fit substantially. Although here was a mismatch in the intermediate phase between the estimated breakpoint and the day the

animal was transferred to uncontaminated medium, with the estimated breakpoint model we were able to show that the beetles switched to decontamination as soon as 2-5 days of intoxication. It should be stressed, however, that the estimated breakpoint model is almost certainly a great oversimplification, not fully justified in terms of the physiology of metal assimilation and excretion. Effectively, this model assumes that metal assimilation stops completely after the maximum concentration is reached. This does not seem very probable because some metal ions are almost certainly assimilated as long as the animals remain exposed to metal-contaminated food. It may happen, however, that the assimilation rate is kept very low thanks to, for example, massive replacement of gut epithelial cells, and that excretion dominates metal toxicokinetics from that moment. The most complex three-phase model described by Laskowski et al. (*submitted*) may supply more insight into temporal changes in the physiology of metal regulation. Because such a model requires estimation of many parameters, more data need to be gathered for such a model to be tested formally. Nevertheless, the preliminary results indicate that this model gives the best description of Ni toxicokinetics in this study (see Fig. 7). The three-phase model assumes that in the first phase of the exposure to metal an animal assimilates the toxicant at a high rate k_a, which can (but might not) be partly balanced by the animal's ability to excrete the toxicant at a rate k_{e1}. There is, however, a certain equilibrium concentration A which an animal can maintain for a prolonged time. When the equilibrium concentration is exceeded (the initial "overshoot"), the animal enters at time D (the switching point), with some lag time, another physiological state at which metal assimilation decreases or even stops. This, together with increased excretion at rate k_{e2}, means that the internal concentration declines to equilibrium concentration A, where it then remains for as long as the animal is exposed to contaminated food (phase 2). Finally (phase 3), when the animal moves to uncontaminated food or is transferred to it by an experimenter, final elimination takes place and the toxicant concentration in the body gradually decreases from an asymptotic equilibrium concentration A to a final concentration C_f which is not necessarily equal to initial body concentration C_0 and for some chemicals it can be zero.

In terrestrial invertebrates, accumulation of metals is avoided by three mechanisms which may occur together in a single species: behavioural avoidance of contaminated substrates, compartmentalization (induction of metal-binding proteins and the formation of a variety of insoluble metal-containing granules), and/or excretion (Hopkin, 1989). In some species a large portion of accumulated metal in the midgut is bound to metallothionein-like protein, and the presence of metal-containing mineral concretions in midgut epithelium (granules) has been demonstrated in several insect species; as these granules may be expelled into the gut lumen by exocytosis or degeneration of whole cells, metal excretion can increase with the efficiency of these processes (Dallinger, 1993). Here, the mechanisms of Ni excretion was not investigated but it cannot be ruled out that a high percentage of the assimilated Ni was excreted rapidly through repeated renewal of the intestinal epithelium affected by necrosis. From a study on *Chrysolina pardalina* feeding on the hyperaccumulating plant species *Berkheya coddii* it is known that the Ni concentrations were highest especially in the Malpighian tubules, which are responsible for elimination of excess Ni from haemolymph, and in the midgut (Przybyłowicz et al., 2002). Those midgut cells showed a specific adaptation of cells forming the tubercles, which are possible sites of stem cells responsible for midgut cells regeneration; excess Ni packed in the vacuolar spaces of these cells is eliminated with them to the lumen of the gut and finally excreted with faeces (Przybyłowicz et al., 2002). No data about average life span of midgut epithelial cells in *P. oblongopunctatus* were found; in the beetle *Tenebrio molitor* it is reported to be four days (Lindqvist et al., 1995) – a value close to the estimated the large variation in Ni levels, seen especially in the first few days of the uptake phase when the beetles were sampled more often, resulted from individual differences in the discharge of gut epithelium cells that took up excessive amounts of the metal. Their rapid discharge to the intestinal lumen and excretion is probably the first direct response to high Ni intoxication. Probably this process was stabilised over the next few days by physiological acclimation, involving decreased assimilation of the metal and/or increased excretion efficiency. These two processes could not be distinguished in this study.

Besides the discharge of gut epithelial cells, removal of significant amounts of Ni with moulting skin probably intensified the elimination of metal in larvae; thist may explain why, unlike adults, larvae were able to depurate down to initial concentrations. Directly before the moult, the metal burden of an animal can be partitioned between the gut epithelium and body (Posthuma et al., 1992). Posthuma et al. (1992) and Sterenborg et al. (2003) attributed high variability of internal cadmium concentrations found in the springtail *Orchesella cincta* to such a pulsed elimination behaviour. There was no assessment of larvae stage in the present study because the moulted skin decomposed in the wet peat, but some differences in k_a and/or D between animals bred at different temperatures might be attributable to asynchronicity of moulting. This would still not explain the temporal pattern of Ni kinetics observed in *P. oblongopunctatus* larvae: the classic one-compartment model could be fitted satisfactorily for springtails in spite of high variability of internal cadmium concentrations (Sterenborg et al., 2003), this was not always possible for Ni in the beetles larvae.

Size-related factors (growth rate, age, body size) can significantly influence accumulation of metals. Apart from a few random incidents, in this study the average body mass change index tended to decrease only in beetles fed 30 mg CPF kg^{-1} (Experiment III), so body mass changes could not have been a significant factor in the observed Ni accumulation and elimination pattern and the high variability of metal concentration in the adult beetles. Moreover, concentrations rather than body burden were used in order to correct for random differences in mass (Jansen et al., 1991). Nor does it seem probable that he large variance in Ni body concentrations, especially during the uptake phase, resulted from short-term variability of food consumption. In the real world, of course, beetles may not eat at a constant rate and may have periods of high ingestion (Brunsting, 1981), but from observations in laboratory studies it is known that when food is constantly available they eat it at a steady rate.

Data on the effect of temperature on metal kinetics in terrestrial invertebrates are scarce. The only direct evidence of a temperature-mediated effect on cadmium uptake and elimination kinetics is given by Janssen and Bergena (1991): 20°C was shown to

increase cadmium accumulation in the collembolan *Orchestella cincta* versus at 10°C, but increased assimilation was matched by increased excretion, resulting in similar body burdens at two temperatures; in the oribatid mite *Platynothrus peltifer* the assimilation rate increased with temperature but the elimination rate was not temperature-dependent, resulting in higher body burdens at 20°C. In two ant species, *Lasus ahenus* and *Formica pratensis*, Nielsen and Jensen (1977) found different increases in cesium elimination rates with rising temperature. They suggested metabolic activity as the major factor influencing the elimination rate in those species.

Why no significant effect of temperature on the assimilation and elimination rates in the adult beetles was found in this study? Perhaps, this was due to the high variation of Ni concentration together with efficient elimination rates already in the uptake phase. However, because the differences in the estimated parameters were quite large (0.009 – 0.026 for assimilation rate k_a, and 2.1 – 7.0 for breakpoint day D, as estimated from weighted regressions), it cannot be excluded that temperature influences Ni kinetics considerably in the real world. Larger sample sizes would probably allow to tell whether these differences are accidental, or would point to real, significant differences in Ni kinetics. The increased sensitivity of *P. oblongopunctatus* to Ni under high temperature observed in previous studies (Chapters 3 and 4) might be due to mechanisms other than changes in assimilation and elimination rates. For example, in the carp *Cyprinus carpio* (L.), Hattink et al., 2005 found hat higher sensitivity to cadmium under hypoxia (i.e., temporary depletion of oxygen) was not explained by a higher Cd body burden initiated by a higher assimilation rate or lower elimination rate under hypoxia. Although the ventilation rate and, in consequence, the total cadmium flow over the gills increased under hypoxia, Cd assimilation was limited by transport across the gill epithelium and was not affected by this higher flow.

Temperature affected growth of larvae, a factor that has to be taken into account when studying metal toxicokinetics in larvae. Increasing body mass affects toxicokinetics by dilution, an effect that can be ignored in adult individuals. It can, significantly affect comparisons of metal toxicokinetics in larvae living at different temperature, as the dilution effect differs between temperatures if the growth is affected by temperature, as

is normally the case. The present data indicate that not all differences in k_a and/or D between temperatures resulted from differences in larval growth: while differences between 10 and 15°C or 10 and 20°C can indeed be attributed to differences in larval growth rates, the difference between 20 and 25°C certainly cannot, since neither the larval specific growth rate g nor final mass M_∞ differed significantly between the two temperatures. On the other hand, despite the growth rate differences, the kinetics parameters at the two temperature extremes did not differ significantly.

Nickel and chlorpyrifos represent completely different groups of chemicals which should exhibit different modes of action and independent effects; metals act through a range of nonspecific effects on redox potential, and the organophosphate pesticides (OP) interact with acetylcholinesterase (AChE). The results of this study suggest that the negative joint effect of Ni and CPF on beetles observed in previous experiments (Chapter 3) cannot be explained by a higher Ni assimilation rate and/or lower elimination rate under CPF exposure, and seem to confirm that the effects of the studied chemicals in combination are independent. Broerse et al. (2009) used the model of Baas et al. (2007) to analyse combined kinetic parameters (no-effect concentration, killing rate and elimination rate); they showed no interaction between Ni and phenanthrene in the springtail *F. candida*. It is known, however, that some metals (arsenic, cadmium) in combination of OPs may exert synergistic effects on AChE inhibition (Forget et al., 1999). In view of this, it is planned to make AChE measurements in beetles sampled along with others taken for Ni kinetics analyses; this will verify the independent action of the chemicals studied here.

Conclusions

All three experiments showed an unexpected behavior of Ni in metal-exposed beetles, with initial rapid accumulation ("overshoot"). It is possible that the ground beetles avoided accumulating excessive Ni levels by rapid discharge of assimilated Ni a few days after the start of exposure, but to fully understand the physiological and

biochemical mechanisms behind this phenomenon, detailed knowledge about detoxification of this metal at the cellular level is needed. With the available data it is impossible to tell whether the decrease in Ni concentration already during the uptake phase resulted from a decreased metal assimilation rate (decreased k_a) or an increased metal elimination rate (k_e), or both working together. Nevertheless, we showed that at least in some circumstances (for particular metals, species and food sources) the classic two-phase toxicokinetic model is not adequate. Although plausible because of its simplicity, it probably omits some important physiological mechanisms that allow organisms to maintain body metal concentrations at a reasonable level even when exposed to extremely polluted food. Such unexpected Ni kinetics together with the large scatter of Ni body concentrations, especially during the uptake phase, may explain the lack of temperature effect on the Ni kinetic in adults and the lack of clear evidence for effects in larvae; since temperature has such a profound effect on the physiology and metabolism of polikilotherms, it was expected that increase in temperature would generally favor higher uptake and/or higher elimination.

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SUMMARY

Due to requirements for standardization and reproducibility, most of ecotoxicological tests are performed under uniform conditions, including constant temperature, usually close to optimal for a test organism. Because of large climatic differences between different regions of Europe (and even more so for the whole World), results of such standard tests can be reasonably representative for only a narrow strip of Earth where average climatic conditions resemble those used in ecotoxicological tests. The combined chemical and physical stressors may cause effects that are greater than or smaller than those caused by single stressors alone. In cases where natural environmental conditions at suboptimal levels enhance toxic effects of chemicals and exacerbating conditions are likely to occur together, more complex experimental designs should be incorporated into ecological risk assessment procedures.

The main aim of this study was to measure effects of interactions between chemicals with different mode of action (nickel and chlorpyrifos) and natural factor (temperature) on the ground beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae). The study is one of the very few existing studies on higher-level interactions between different stressors (Chen et al., 2004, 2008; Heugens et al., 2006), and the only one in which the data were used to estimate multiple effects of two different chemicals at a range of temperatures in terrestrial invertebrates.

In the first part of this thesis (Chapter 2) the survival, respiration rates and internal body Ni concentrations in beetles exposed to Ni for a long period (245 d) were studied. Nickel concentration in the beetles increased steadily with increasing concentration in food with LC_{50} estimated at 8351 mg Ni kg^{-1} and with no effect on fertility. The negative correlation between Ni exposure and respiration rate was found. However, when the beetles were exposed to 2400 mg Ni kg^{-1} (LOEC for the respiration rate) for a shorter period (64 d) at temperatures 10 and 20°C, their metabolic rates either did not differ from control beetles or the effect was minor and statistically significant only in beetles living at 20°C. This means that effect of Ni on beetles respiration rate depends on time of exposure, what was also confirmed in further study, not included in this thesis (Bednarska et al., 2008; Bednarska et al., *submitted*). Moreover, it became clear that the negative effects of Ni may appear after relatively long exposure to high levels

of the metal. The significant hormetic effect suggests that the beetles may require Ni at somewhat elevated levels. Based on the results presented in Chapter 2, the concentrations of Ni and time of exposure were chosen for further three-factor experiments (Chapter 3) as well as for Ni toxicokinetics study (Chapter 5).

The hypotheses regarding effects of interactions between the three factors: Ni, chlorpyrifos (CPF) and temperature were tested in a full-factorial design with at least three levels of each factor. Separate experiments were run on adult beetles (Chapter 3) and on larvae (Chapter 4), and the pathway of exposure to chemicals were adjusted to developmental stage of beetles to reflect environmentally realistic scenarios. The results showed that temperature interacts not only directly with chemicals but also affects interactions between chemicals – adult beetles were less sensitive to combined effect of the chemicals at lower temperature (10°C) than at higher (25°C). Similarly, larvae survived better if they were exposed to combined chemicals at 10°C than at 25°C. In contrast to survival data, no second order interactions were found in fecundity (expressed as a number of eggs per female) or in development success rate (expressed as proportion of emerged imagines). In both cases only the first order interactions were identified: the reproduction was most sensitive to Ni concentration at the lowest and highest temperatures, while the effect of Ni and temperature on the proportion of emerged imagines indicated stronger toxicity of Ni at higher temperature. These results show that different endpoints may be differently sensitive to the same interaction and underline the importance of considering multiple factors in assessment of risk brought by exposure to toxic chemicals in natural conditions.

In Chapter 5 the questions were raised to what extent the observed interactions resulted from different accumulation and/or elimination kinetics of Ni at different temperatures, and to what extent CPF influenced Ni toxicokinetics and, thus, its toxicity. Moreover, the analysis of metal accumulation and elimination patterns might give useful information on probable Ni detoxification mechanisms. Because beetles do not regulate their body temperature, all physiological and biochemical processes depend on external temperature. Therefore, differences in Ni uptake and/or elimination kinetics were expected between different thermal conditions. Contrary to expectations, no clear

temperature effect on Ni toxicokinetics was found in both adults and larvae. Moreover, neither assimilation nor elimination of Ni was affected by CPF. One of the possible causes of this lack of detectable effects of temperature or CPF could be the unexpected three-phase rather than two-phase kinetics of this metal. Unfortunately, statistical significance of parameters and possible differences between treatments could not be tested statistically due to limited amount of data. Based on the observed pattern, it can be hypothesized, however, that Ni stored within vesicles of digestive cells is simply released together with whole degenerated gut epithelial cells after a necessary time-lag and the dead cells are then replaced with new ones. It is also possible that changes in toxicokinetic parameters are linked to changes in the turnover rates of proteins involved in the transport of the metal ions. Both hypotheses need to be verified in future more detailed histological and/or biochemical studies. Whatever the mechanism, in case of this study the classic two-phase toxicokinetic model was not adequate; it probably omits some important physiological phenomena that allow organisms to maintain body metal concentrations at reasonable level even when exposed to highly polluted food. The three-phase model that allows for different assimilation and/or elimination rates in different phases of exposure to a toxicant may provide more insight into temporal changes in the physiology of metal handling and should be tested when temporal patterns of internal metal concentration exhibits an initial "overshoot" in body metal concentrations.

Ecological risk assessment of multiple stressors is not sufficiently developed yet; as a consequence, there is a large demand for knowledge on effects of interactions between chemicals with different modes of action and suboptimal environmental conditions. The experiments described herein revealed the complexity of interactions between natural and anthropogenic factors in their combined effects on terrestrial organisms. Of course, the interaction patterns described in this thesis lack generality and cannot be translated yet to other mixtures, species, etc., without mechanistic explanation of combined effects of different types of stressors. From that point of view, a promising approach is the use of relevant biomarkers. Therefore, to fully confirm independent action of the chemicals studied and to better understand toxicant behaviour,

measurements of activity of selected enzymes (e.g., AChE and glutathione-S-transferase, GST) in the beetles sampled simultaneously with individuals used for Ni toxicokinetics study are planned. AChE is widely used as a biomarker to diagnose OPs poisoning, and GSTs are family of enzymes involved in detoxification of a broad range of xenobiotics, including pesticides.

PODSUMOWANIE

Niniejsza praca prezentuje wyniki badań poświęconych interakcjom pomiędzy substancjami chemicznymi oraz pomiędzy substancjami chemicznymi i temperaturą i ich wpływowi na bezkręgowce glebowe. Badanymi czynnikami chemicznymi były: nikiel (Ni), jako przedstawiciel metali, i chloropiryfos (CPF) – pestycyd z grupy związków fosforoorganicznych. Wszystkie eksperymenty składające się na tę rozprawę przeprowadzone zostały na organizmie modelowym – chrząszczu *Pterostichus oblongopunctatus* (Coleoptera: Carabiade).

W naturalnym środowisku organizmy nigdy nie są narażone na działanie tylko jednego czynnika stresowego, gdyż ani zanieczyszczenie nigdy nie jest obecne w przyrodzie w postaci pojedynczej substancji chemicznej, ani same organizmy nigdy nie przebywają stale w optymalnych dla siebie warunkach biotycznych i abiotycznych. Dlatego badania nad wpływem interakcji pomiędzy samymi substancjami chemicznymi oraz pomiędzy nimi a czynnikami naturalnymi, zarówno na organizmy wodne jak i lądowe, są obecnie w centrum zainteresowania ekotoksykologii. Z roku na rok przybywa prac związanych z tym zagadnieniem, jednak dotyczą one głównie układów dwuczynnikowych, przy czym gros prac koncentruje się na interakcjach pomiędzy samymi substancjami chemicznymi. Oryginalny wkład w ten nurt, jak wnosi niniejsza rozprawa, polegał na zbadaniu skutków jednoczesnego narażenia organizmów na kilka czynników o różnym sposobie działania, w tym naturalnych czynników środowiskowych. Problem ten nie był dotychczas badany u bezkręgowców glebowych. Równie ważnym elementem pracy są eksperymenty toksykokinetyczne, przeprowadzone w celu zbadania mechanizmu odpowiedzialnego za istotne interakcje pomiędzy badanymi czynnikami. Zagadnienia te były przedmiotem kilku oddzielnych, acz komplementarnych, eksperymentów. Ponieważ każdy z nich stanowił integralną część, ich wyniki zostały przedyskutowane osobno. Miały one jednak wiele wspólnych elementów, co pozwoliło na wyciągnięcie wniosków w szerszym kontekście.

Za względu na skromne dane na temat toksyczności niklu dla bezkręgowców glebowych, w Rozdziale 2 zbadano zdolność chrząszczy do akumulacji tego metalu oraz wpływ Ni na przeżywalność i tempo respiracji chrząszczy. Wraz ze wzrostem stężenia Ni w pokarmie rosło stężenie tego metalu w ciele chrząszczy. Odwrotna

zależność została stwierdzona w przypadku tempa respiracji: długoterminowa ekspozycja na podwyższone stężenia Ni pociągała za sobą spadek tempa procesów metabolicznych. Nikiel okazał się metalem mało toksycznym dla chrząszczy (LC_{50} = 8351 mg Ni kg^{-1}), chociaż przy stężeniu 2400 mg Ni kg^{-1} (LOEC) zaobserwowano istotny spadek tempa respiracji. Niemniej jednak, gdy chrząszcze eksponowano na wspomniane stężenie przez trzykrotnie krótszy okres (64 dni) w temperaturze 10 i 20°C, tempo ich metabolizmu różniło się w porównaniu do chrząszczy z kontroli tylko nieznacznie, a statystycznie istotne różnice zaobserwowano jedynie w przypadku temperatury 20°C. Otrzymane wyniki pokazują, że negatywny wpływ Ni na tempo respiracji zależy między innymi od czasu ekspozycji, co również zostało potwierdzone w późniejszych badaniach (Bednarska et al., 2008; Bednarska et al., *złożone do druku*). Z kolei stwierdzona równocześnie istotna hormeza sugeruje, że w niższych stężeniach Ni może być chrząszczom niezbędny i w naturalnych stężeniach jest czynnikiem ograniczającym. W oparciu o wyniki przedstawione w Rozdziale 2 ustalono stężenia Ni i czas ekspozycji do dalszych eksperymentów na dorosłych chrząszczach (Rozdziały 3 i 5).

Celem badań opisanych w Rozdziałach 3 i 4, a zarazem głównym celem niniejszej rozprawy, było zbadanie wpływu interakcji pomiędzy Ni, CPF i temperaturą na podstawowe cechy historii życiowej *P. oblongopunctatus*. Aby dostosować warunki eksperymentu do tych panujących w terenie, zarówno dorosłe chrząszcze jak i larwy eksponowano na Ni przez pokarm (chociaż na różne stężenia), jednak sposób aplikacji pestycydu był różny: osobniki dorosłe eksponowano na pestycyd poprzez jego aplikację na powierzchnię ciała, natomiast larwy eksponowane były poprzez skażone podłoże. W obu eksperymentach zastosowano te same temperatury: 10, 20 i 25°C. W Rozdziale 3 opisano bardzo wyraźny wpływ temperatury nie tylko na toksyczność pojedynczych substancji chemicznych, ale także na interakcje pomiędzy badanymi substancjami, o czym świadczy statystycznie istotna interakcja drugiego stopnia. Podobne wyniki uzyskano w eksperymencie na larwach (Rozdział 4), gdzie również stwierdzono, że chrząszcze przeżywały lepiej, jeśli były eksponowane na Ni i CPF w niższej temperaturze (10°C), niż w wyższej (25°C). Natomiast nie stwierdzono

wpływu interakcji drugiego stopnia na inne niż przeżywalność cechy historii życiowej. Stało się również jasne, że różne cechy są wrażliwe na różne interakcje. Przykładowo, reprodukcja była parametrem najbardziej wrażliwym na Ni w skrajnych temperaturach, podczas gdy negatywny wpływ Ni na proporcję osobników dożywających do imago wzrastał wraz ze wzrostem temperatury.

Interakcja trzyczynnikowa pomiędzy Ni, CPF i temperaturą pokazała, ze chrząszcze były mniej wrażliwe na łączne działanie badanych substancji w niższej temperaturze. Jednak bez szczegółowych badań nie można powiedzieć czy działo się tak dlatego, że na przykład chrząszcze akumulowały mniej Ni w niższej temperaturze. Równie trudno byłoby odpowiedzieć na pytanie, czy CPF wpływał na akumulację Ni, a przez to na jego toksyczność, czy też może jego wpływ był zupełnie niezależny od wpływu metalu, a za wzrost toksyczności w interakcji odpowiada raczej nagromadzenie się skutków niezależnego działania obu substancji. Odpowiedź na tego typu pytania umożliwiają eksperymenty toksykokinetyczne, których wyniki zostały przedstawione i omówione w Rozdziale 5. Analiza tempa akumulacji i eliminacji Ni może dostarczyć również ważnych informacji na temat prawdopodobnych mechanizmów detoksykacji badanego metalu. Ponieważ chrząszcze nie są zdolne do regulacji temperatury ciała, wszystkie procesy fizjologiczne i biochemiczne zależą od temperatury zewnętrznej. Dlatego spodziewano się różnic w stałych akumulacji i/lub eliminacji Ni pomiędzy badanymi temperaturami (10; 15; 20; 22,5; 25°C). Niestety, na podstawie uzyskanych wyników nie da się jasno stwierdzić, czy takie różnice w rzeczywistości istnieją; w eksperymencie przeprowadzonym na osobnikach dorosłych nie stwierdzono różnic w stałych akumulacji i eliminacji pomiędzy badanymi temperaturami, natomiast w eksperymencie na larwach istotne różnice były, jednak nie układały się one w żaden klarowny trend. Nie wykazano także wpływu CPF na stałe akumulacji i eliminacji Ni. Zaobserwowano jednak, że niezależnie od temperatury i stężenia CPF chrząszcze rozpoczynały efektywną eliminację Ni jeszcze podczas fazy intoksykacji. To nietypowe zachowanie Ni nie pozwoliło na zastosowanie klasycznego modelu toksykokinetycznego, powszechnie używanego w badaniach toksykokinetycznych. Dlatego podjęto próbę opracowania bardziej skomplikowanego modelu, lepiej

odzwierciedlającego toksykokinetykę badanego metalu u *P. oblongopunctatus*. W tym celu zastosowano model z estymowanym punktem przełączenia. W modelu tym pominięto rozróżnienie pomiędzy okresami akumulacji i eliminacji i zastosowano procedurę szacującą dzień przełączenia organizmu na dekontaminację na podstawie uzyskanych danych o wewnętrznych stężeniach metalu. Model taki jest jednak bardzo dużym uproszczeniem, gdyż dane wskazują na raczej trójfazową niż dwufazową toksykokinetykę. Niestety, ze względu na brak wystarczająco obfitych danych i zbyt dużą wariancję międzyosobniczą w stężeniach Ni, model trójfazowy i ewentualne różnice w parametrach takiego modelu pomiędzy zabiegami nie mogły być przetestowane pod względem statystycznym. Ponieważ Ni jest pierwiastkiem bardzo słabo do tej pory zbadanym od strony ekotoksykologii, brak prac na temat kinetyki tego metalu znacznie utrudnia interpretację otrzymanych wyników. Konieczne są dalsze badania nad tym metalem, aby móc stwierdzić, w jaki sposób jest on unieszkodliwiany i usuwany z organizmu. Wydaje się bardzo prawdopodobne, że za nietypową toksykokinetykę odpowiada po prostu usuwanie Ni ze złuszczającymi się komórkami nabłonka jelita, które następnie są zastępowane nowymi komórkami. Możliwe jednak, że zmiany w parametrach toksykokinetycznych są związane ze zmianami w szybkości obrotu metabolicznego białek związanych z transportem jonów Ni. Zweryfikowanie obu hipotez wymaga dalszych, bardziej szczegółowych badań histologicznych i biochemicznych. Niemniej jednak, niezależnie od mechanizmu stojącego za nietypową kinetyką Ni, w badaniach prezentowanych w niniejszej rozprawie pokazano, że model klasyczny jest zbyt dużym uproszczeniem, gdyż prawdopodobnie nie uwzględnia ważnych fizjologicznych zjawisk, które umożliwiają utrzymanie stężenia metalu w ciele na względnie niewysokim, stabilnym poziomie nawet podczas ekspozycji na silnie skażony pokarm. Model trójfazowy, uwzględniający różne stałe akumulacji i/lub eliminacji w różnych fazach ekspozycji na substancje toksyczną, pozwala na lepszy wgląd w zmiany fizjologii metalu w czasie i powinien być testowany za każdym razem, gdy dane wskazują na gwałtowny wzrost wewnętrznego stężenia metalu w początkowym okresie intoksykacji, a następnie spadek tego stężenia, pomimo trwającego nadal narażenia na wysokie stężenia metalu. Założenia modelu trójfazowego zostały przedyskutowane w Rozdziale 5.

Szacowanie ryzyka ekologicznego powodowanego przez wieloczynnikowy stres nie jest obecnie wystarczająco rozwiniętą działalnością, wobec czego brakuje wiedzy na temat skutków interakcji pomiędzy substancjami chemicznymi o różnym sposobie działania i suboptymalnymi warunkami środowiska. Opisane w niniejszej rozprawie eksperymenty wskazują na skomplikowany wpływ interakcji pomiędzy naturalnymi i antropogenicznymi czynnikami na organizmy. Oczywiście, ze względu na brak jasnego wyjaśnienia mechanizmu odpowiedzialnego za zaobserwowane interakcje, uzyskane wyniki nie mogą być uogólnione na interakcje pomiędzy innymi czynnikami czy na gatunki. Aby w pełni odpowiedzieć na pytanie dotyczące sposobu działania Ni i CPF w mieszaninie, obiecujące wydaje się być użycie odpowiednich biomarkerów (np. aktywność acetylocholinesterazy lub transferazy-S-glutationowej). Próby zebrane podczas eksperymentu opisanego w Rozdziale 5 będą dobrym materiałem do wykonania tego typu analiz.

Podsumowując, chociaż eksperymenty badające wpływ wieloczynnikowego stresu są skomplikowane, a uzyskane wyniki nieraz trudne do interpretacji, badania tego typu są niezbędne w celu lepszego ekstrapolowania wyników uzyskiwanych w laboratorium na warunki panujące w terenie.

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