Stellingen

1. Het gebruik van hsp60 als biomarker kan niet leiden tot een valide uitspraak omtrent het ecotoxicologische effect van chemische stoffen; een goede interpretatie kan alleen verkregen worden indien hsp60 wordt opgenomen in een reeks van andere biomarkers.

   Dit proefschrift

2. Het verloop van de hsp60 respons bij toenemende stress wordt correct beschreven door een optimum curve en niet door de klassieke dosis/concentratie respons curve.

   Dit proefschrift

3. De termen stress eiwitten en heat shock proteins worden vaak –ten onrechte- door elkaar gebruikt.

   Hightower (1993) *Marine Environ. Res.* 35: 79-83.

4. Voor ecotoxicologische risico-analyses hebben multigeneratie experimenten een duidelijke meerwaarde vergeleken met de enkele generatie experimenten door de detectie van mogelijke trade-offs tussen levenscyclusonderdelen en fitness consequenties op langere termijn.

   Dit proefschrift

5. Mensen en een aantal pathogenen hebben gemeenschappelijk dat ze hun leefomgeving eerst koloniseren, dan exploiteren ten behoeve van zichzelf en uiteindelijk in een deplorabele staat achterlaten.

6. No one sees the man who climbs the mountain until he reaches the top

   Shona Ama

7. A man who knows how little he knows is well, a man who knows how much he knows is sick.

   Lao Tzu

8. Voor de meeste d.j.'s op radio 3 is het ten gehore brengen van muziek slechts een bijzaak.

Stellingen behorend bij het proefschrift getiteld: 'Metal stress in free-living nematodes', door Marie-José Arts.

Wageningen, 21 december 2001

METAL STRESS IN FREE-LIVING NEMATODES

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METAL STRESS IN FREE-LIVING NEMATODES

Maria Silvestra José Hamelijnck-Arts

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 21 december 2001
des morgens om 11.00 uur in de Aula

Arts, M.S.J.

Metal stress in free-living nematodes

CIP-DATA Koninklijke Bibliotheek, Den Haag

Thesis Wageningen University and Research Centre– with references – with summaries in English and Dutch
ISBN 90-5808-533-3
Subject headings: free-living bacterivorous nematodes/ metals/ toxicology/ biomarker/ hsp60/ fitness consequences

CONTENTS

Chapter 1: Introduction.....7

Chapter 2: Hsp60 as a potential biomarker of toxic stress in the nematode *Plectus acuminatus*.....21

Chapter 3: Identification of hsp60 in the nematode *Plectus acuminatus* and its quantitative response to metal exposure.....31

Chapter 4: Application of the hsp60 biomarker in the nematode *Plectus acuminatus* exposed in the field to metals in a gradient near Avonmouth, UK.....47

Chapter 5: Multi-generation cadmium effects at the population level: Fitness maximisation and optimal allocation in the nematode *Caenorhabditis elegans*.....61

Chapter 6: Discussion and conclusions.....75

Summary.....85

Samenvatting.....91

Dankwoord.....97

Publications.....99

Curriculum vitae.....101

CHAPTER 1

Introduction

The term 'ecotoxicology', as introduced in 1977 by Truhaut, reflects a growing concern about the effects of environmental chemicals upon species other than man. Ecotoxicology roots from several disciplines like toxicology, ecology, biochemistry and environmental chemistry and aims to detect, monitor and predict the effects of contaminants on ecosystems (Forbes and Forbes, 1994). Whereas classical toxicology focuses upon effects of xenobiotics on the individual level and their sites of action, ecotoxicology is concerned with the ultimate effects at higher organisational levels such as populations and ecosystems.

Short-term acute toxicity tests provide information on mortality effects. However, these tests have little or no predictive value for chronic exposure at lower dose levels. Realising that mortality is too coarse a measure for toxicity, alternatives have been explored and new methodologies developed. Apart from sublethal effects on organisms, such as inhibition of growth or reproduction, biomarker responses are now often used as ecotoxicological endpoints.

1.1 The use of biomarkers

Detection of biomarkers provides a powerful tool for the early assessment of exposure and/or effect of environmental contaminants at the below-individual level (Van Gestel and Van Brummelen, 1996). Changes at the biochemical level are usually the first detectable responses to environmental perturbation. Because these alterations underlie all effects at higher organisational levels (see Fig.1), they can be helpful tools in ecotoxicological risk assessment. In line with the ecotoxicological literature, we focus the term biomarker further to the detection of molecular, biochemical, physiological or cellular alterations in organisms following exposure to pollutants (Peakall and Shugart, 1992; Deplegde and Fossi, 1994).

Biomarkers of exposure are those that indicate exposure of the organisms to xenobiotic agents, but do not give information about the degree of adverse effect that this change causes. Biomarkers of effect are those which demonstrate an adverse effect on the organism or can be associated with an established or possible health impairment or disease. The fact that the relationship between the biomarker of exposure and an adverse effect is not clear-cut does not invalidate the use of that biomarker. A biomarker can answer the question if there is an environmental pollutant or pollutants present at a sufficiently high concentration to cause an effect. If the answer is positive, further investigation to assess the nature and degree of damage and the causal agent or agents is justified. If negative, it means that additional

resources do not have to be invested. In this way biomarkers can act as an important 'early-warning' system. Both specific and non-specific biomarkers are valuable in environmental risk assessment. Non-specific biomarkers can assess the health of the organism in general (Walker *et al.*, 1996).

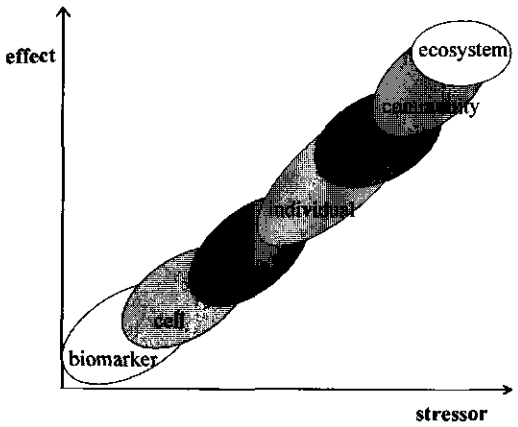


Figure 1: Schematic representation of different organisational levels in ecotoxicology, varying from very low (biomarker) to the highest (ecosystem) with increasing ecological relevance.

A major advantage of the application of biomarkers is that they tend to be more sensitive, more highly conserved and often easier to measure than stress indices commonly examined at the organismal level, such as inhibition of growth, changes in rate of development and reduced reproduction (Sanders, 1990). Biomarkers have the advantage of measuring the actual effective fraction of pollution that affects an organism by integrating multiple exposure routes over a given time interval and for any given number of pollutants (Bierkens, 2000). However, there are some limitations, inherent in the use of biomarkers as indicators of toxic stress, such as (i) the sensitivity of the biomarker response to various natural biotic and abiotic conditions, which are referred to as confounding factors, and (ii) the transiency of the biomarker response. After exposure to contaminants, the initial induction of some biomarkers may fade away, thus obscuring the applicability of biomarkers in the field to assess the history of exposure or effect. On the other hand, other biomarkers may be induced for extended periods as long as the exposure continues, thus enhancing field applicability. Most research on biomarkers focuses on understanding the responses at lower levels of biological organisation such as molecules, cells and individuals. However, contaminants can exert their effects at all levels of biological organisation from molecules to ecosystems (see Fig. 1). Because it is very difficult to make a substantive linkage between biomarker responses and changes at higher orders of biological organisation, it is advocated that one seeks for probable correlations between a biomarker response and an individual- or population-level effect (Kammenga *et al.*, 2000). For instance, a relationship was found between biomarker responses and demographic parameters under laboratory conditions (Köhler *et al.*, 1998). In the field these correlations are extremely difficult to reveal, because one should recognize the biomarker response transiency, its normal fluctuation under ambient conditions and the influence of climatic variations and life stage (Kammenga *et al.*, 2000).

1.2 Stress proteins

If we select as biomarker cellular and biochemical events which are intimately involved in protecting and defending the cell from environmental insults, we have ideal candidates for biomarkers of exposure and possibly of effect (Sanders, 1990). Cells dramatically alter their gene expression in response to environmental stress, attempting to protect themselves from damage and to repair existing damage (Schlesinger *et al.*, 1982). This response is called the cellular stress response and it can be elicited by a variety of chemical, physical and biological stressors. Changes in gene expression associated with the stress response are extremely rapid and result in the induced synthesis and accumulation of stress proteins.

The stress proteins can be divided into three major groups: (i) the heat shock proteins (hsps) whose synthesis is dramatically increased by temperature shock and a variety of other stressors, (ii) the glucose regulated proteins (grps) whose synthesis is increased in cells deprived of either glucose or oxygen, (iii) stressor-specific stress proteins, induced by a specific set of chemical or physical conditions and not substantially increased by heat. Hsps and grps are closely related, having similar biochemical and immunological characteristics and sharing considerable homology between families. In many instances, the synthesis of these two groups of stress proteins seems to be inversely regulated, e.g. cells deprived of glucose have increased synthesis of grps and a concomitant decreased synthesis of hsps. Stressor-specific stress proteins are part of the cell's immediate stress response, but not part of the cell's protective system in response to general cellular damage. As a consequence, this group of stress proteins does not encompass a homologous group of proteins and is not related functionally or structurally to either of the two other groups of stress proteins. To the stressor-specific stress proteins belong the metallothioneins and the enzyme heme oxygenase (Sanders, 1990).

1.3 Heat shock proteins (hsps)

The majority of papers published on hsps deal with diagnostic or prognostic studies in the medical field. The heat shock response is a fundamental aspect of cellular physiology in which exposure to a stressor results in a dramatic redirection of metabolism, such that this suite of stress proteins is rapidly synthesized and the synthesis of other cellular proteins is repressed (Lindquist, 1986). The heat shock response was initially discovered in the fruit fly *Drosophila melanogaster* salivary glands upon exposure to elevated temperature (Ritossa, 1962) and identified by Tissières *et al.* (1974). It has been found in all organisms examined to date and the genes which encode the hsps and the hsps themselves are remarkably conserved, from bacteria to man (Schlesinger *et al.*, 1982). Collectively, the hsps comprise a group of stress proteins whose rates of synthesis rise sharply following an increase in temperature to 3-10°C above the ambient temperature of the organism involved (heat shock). However, most hsp families can be induced by a variety of stressors including heavy metals, xenobiotics, teratogens, uv radiation (an overview can be found in Nover, 1991 and Sanders, 1993). Many hsps are constitutively expressed. Their presence in unstressed cells suggests that they play a role in the cell's basic physiology (Sanders, 1990). Some of these constitutively expressed hsps (sometimes called heat shock cognates (hscs)) are upregulated proportionately to the degree of stress (e.g. Sato *et al.*, 1993). Other isoforms (hsp72 being the most prominent among them) are believed to be synthesized only when the cell is exposed to adverse conditions and yet others show a stress-modulated expression (e.g. Mizzen and Welch, 1988; Chen *et al.*, 1992). Both cognates and stress-inducible hsps share common functions as

molecular chaperones (Ellis, 1987). They for example assist in the transport (in an unfolded state across intracellular membranes), folding and assembly of newly synthesized proteins. The same or similar hsps also help to rescue damaged or misfolded proteins and to refold them correctly, while severely damaged proteins are escorted away for proteolysis (Parsell and Lindquist, 1993). The common signal elicited by all hsp-inducing stressors involves an abnormally high concentration of damaged/aggregated proteins within cells, a phenomenon generally referred to as 'proteotoxicity' (Hightower, 1993). Hsp biomarkers give an integrated response summarizing the total proteotoxic damage caused within the target organism or organism tissue. The involvement of chaperones in so many key cellular functions renders the hsp system exquisitely sensitive to many different pertubations.

Although the number of hsps induced by a certain stressor and the exact size of hsps are both tissue and species specific, most classes are found in all eukaryotes. Based on their molecular weight, hsps can be classified into different families: A heterogeneous group of low molecular weight hsps (LMW; 15-40 kDa), a group of about 60 kDa called chaperonins (hsp60, stress-60, cpn60; 55-65 kDa), the family of about 70 kDa (hsp70, stress-70; 66-78 kDa), 90 kDa (hsp90, stress-90; 79-98 kDa) and high molecular weight stress proteins (HMW; 100-110 kDa). Additionally, a small protein of 7 kDa, ubiquitin, which is involved in nonlysosomal degradation of intracellular proteins and a protein of 10 kDa, associated with hsp60, are usually assigned to the hsps. In eukaryotes, each hsp is the member of a multigene family, regulated by different promotors and coding for closely related protein isoforms (Lindquist,1986).

Below the hsp90, hsp70 and LMW hsps will be briefly discussed. The emphasis will lay on the hsp60 family, because this family plays the major role in the present thesis.

Hsp90 (stress-90; 79-98 kDa)

Under normal conditions, hsp90 modulates many cellular activities by binding to target proteins, forming an inactive or unassembled complex (Gething and Sambrook, 1992). These target proteins include enzymes, hormone receptors and components of the cytoskeleton. Upon exposure to environmentally stressful conditions, the synthesis of hsp90 increases and may redirect cellular metabolism to enhance tolerance. However, the specific mechanisms involved have not been identified (Sanders, 1993). Given the normal abundance of hsp90 and its limited induced synthesis upon exposure to stress, it may not have a great deal of potential as a biomarker for general stress (Sanders, 1990).

Hsp70 (stress-70; 66-78 kDa)

The ubiquitous hsp70 family is the most highly conserved and the largest of all the hsp families; it also has been the most extensively studied. Members of the hsp70 family are found in several subcellular compartments. Primarily, they bind to target proteins to modulate protein folding, transport and repair (Sanders, 1993). In addition to these roles in protein homeostasis, hsp70 is also involved in gene regulation through interactions with transcription factors (Panniers, 1994). Under adverse environmental conditions, the hsp70 synthesis increases and some hsp70 members are newly induced (e.g. hsp72) (Mizzen and Welch, 1988). Hsp70 is an excellent candidate for a biomarker for general stress (Sanders, 1990) and has been subjected to many environmental studies.

Hsp60 (stress-60, chaperonin(-60), cpn60; 55-65 kDa)

The chaperonin family is found in eubacteria and in eukaryotic cells, almost exclusively in organelles which are probably of endosymbiotic origin (mitochondria, chloroplasts) (Hemmingsen *et al.*, 1988). Hsp60 is homologous to the bacterial GroEL and the Rubisco Subunit Binding Protein (RuSBP) of chloroplasts (Reading *et al.*, 1989). It is a nucleus encoded, constitutively expressed protein. As a molecular chaperone, hsp60 also binds target proteins to facilitate folding and assembly (see Fig. 2); however, unlike hsp70, binding seems to involve side-chain hydrophobicity (Flynn *et al.*, 1991). As a consequence, the same peptide binds to chaperonin in a conformation different from that of hsp70, suggesting that these two hsp families perform different folding functions. Furthermore, unlike hsp70, which binds to target proteins as a monomer, chaperonin forms an oligomeric complex which is arranged as two stacked heptameric rings of identical or closely related rotationally symmetric 60 kD subunits that form a central cavity (Hendrix, 1979; Hohn *et al.*. 1979; McMullin and Hallberg, 1988). Under normal conditions, this complex binds incompletely folded proteins in its central cavity, directs the folding peptide to the correct conformation and prevents aggregation of incompletely folded proteins until they are competent for oligomer assembly (Gething and Sambrook, 1992). In most cases, ATP and a single heptameric ring of 10 kDa subunits (hsp10) are required for folding of the polypeptide intermediates and release from the chaperonin (Martin *et al.*, 1991). Under adverse environmental conditions that cause an increase in protein denaturation, the synthesis of chaperonin increases. It takes on an additional role during protein repair by binding to damaged proteins to help refold them to their native conformation. Increased levels of chaperonin also can protect against protein denaturation and aggregation (Martin *et al.*, 1992). However, unlike hsp70, chaperonin is not able to break up existing aggregates (Gething and Sambrook, 1992). Hsp60 also facilitates the translocation of oligomeric proteins into the mitochondria and chloroplasts of eukaryotes (Cheng *et al.*, 1989). Because it is highly conserved and its synthesis is increased in stressed cells, it is a good candidate as a biomarker for general stress (Sanders, 1990).

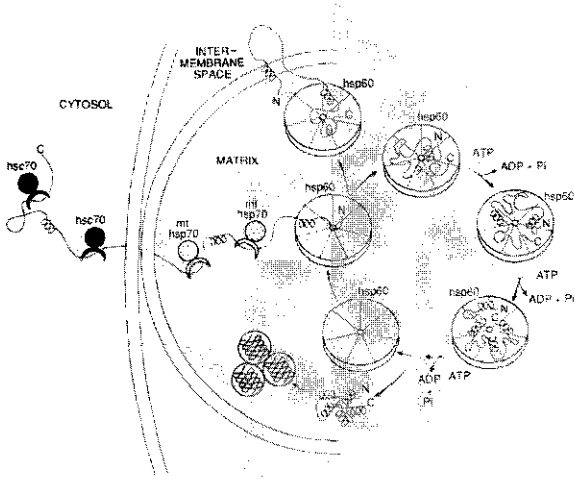


Figure 2: Illustration of the putative roles of chaperonin and hsp70 molecules during the import of mitochondrial precursors, their subsequent folding in the mitochondrial matrix and their reexport to the intermembrane space (Gething and Sambrook, 1992).

The expression of the mitochondrial hsp60 has been subject to biomarker studies concerning soil invertebrates. Sanders *et al.* (1994) mentioned the elevation of the chaperonin level by increased temperature in the earthworm *Eisenia fetida*. Mariño *et al.* (1999) reported upregulation of hsp60 in transplanted but not in resident populations of the earthworm *Lumbricus rubellus* in metal contaminated soils in laboratory experiments. Exposure to metals (zinc, lead and cadmium) slightly increased the hsp60 level in the supernatant of homogenates of the slug *Deroceras reticulatum*, the diplopod *Julus scandinavius* and the isopods *Oniscus asellus* and *Porcellio scaber*, but in comparison to hsp70 the biomarker response to metal exposure was much less sensitive (Eckwert *et al.*, 1997).

Sometimes another protein family that may participate in protein folding in the cytoplasm is assigned to the chaperonin family. Two members of this family have been identified: one is a major heat-inducible protein referred to as TF55, whose synthesis correlates with thermotolerance in the thermophilic archaebacterium *Sulfolobus shibatae*. The other member of this family, t-complex polypeptide 1 (Tcp-1), is an ubiquitous eukaryotic cytoplasmic protein involved in mitotic spindle formation. Remarkably, although there is little amino acid sequence similarity with chaperonin, Tcp-1 forms a large oligomeric complex of two nine-membered rings comprised of identical 55-kDa subunits that is highly reminiscent of the chaperonin complex (Gupta, 1990; Trent *et al.*, 1991). Although the similarity in quaternary structure to chaperonin lends strong support that this protein family may play a role in protein folding and assembly, Tcp-1 synthesis is not induced by heat and at this point it cannot be classified as a cytoplasmic chaperonin (Ursic and Culbertson, 1992).

Low molecular weight hsps (LMWs; 15-40 kDa)

This group of hsps is more diverse, more species specific and less highly conserved compared to the other major hsp families (Nover, 1991). However, all members of this hsp family examined share homology to the α-crystalline lens protein and share with that protein the ability to form higher order structures of approximately 500.000 kDa (Arrigo and Welch, 1987). Unlike hsp90, hsp70 and hsp60, the LMW hsps are not synthesized under normal conditions. Their synthesis is strictly induced under adverse environmental conditions and is regulated during development and differentiation by several hormones (Lindquist, 1986). However, little is known regarding their specific cellular function (Sanders, 1993). Since they are highly species specific and regulated by a number of factors besides exposure to stressors, their use as a biomarker for general stress may be misleading and of limited value (Sanders, 1990).

The sensitivity of the hsp response to induction by a wide variety of chemical and/or physical stressors makes it attractive to ecotoxicologists. However, screening for only one particular hsp family may not provide a sufficiently sensitive bioindicator for a wide range of pollutants, because different agents induce different hsp families and with widely differing efficiencies. Furthermore, some physiological extremes can activate *hsp* genes, leading to elevated expression of hsps in the absence of any deleterious chemical/physical stressors. Therefore, hsp biomarkers need to be evaluated alongside other biomarkers to determine their predictive value and ecological relevance (De Pomerai, 1996). Except for the problems of hsp biomarkers concerning confounding factors, also transiency is a reason not to apply the hsp biomarker solely but rather to include it in a suite of other biomarkers. The inclusion of hsps in a biomarker set is necessary if the aim is also to register the adverse effects of nonchemical stressors (e.g. temperature), which in concert with the chemical pollution determine the actual and therefore ecologically relevant stress situation in field sites (Kammenga *et al.*, 2000).

1.4 Nematodes as target organisms

Terrestrial invertebrates offer meaningful targets for assessing the potential adverse effects of chemicals on soil ecosystems. Invertebrates play a major role in the functioning of the soil ecosystem by enhancing the soil structure, mineralization and the decomposition of organic material. Furthermore, they are an important chain in the foodweb, because they cover many trophic levels: From protozoan, algae, bacterial, fungal and plant feeders to predators. Usually, they serve as a food source for (other) predators. Furthermore, they represent a major component of all animal species in soils and often are present in high population densities (Kammenga *et al.*, 2000).

The most dominant group of terrestrial invertebrates, in fact of all multicellular organisms on earth, are nematodes, also called threadworms or roundworms. Nematodes are usually small (0.2-2 mm), transparent and present in almost every habitat on earth. In soil, they usually occur in high abundances (several million per m^2) and a considerable species diversity (20-60 per sample), depending on soil structure (including organic matter content and pH), texture and vegetation (Sohlenius, 1980). They are representative of soil samples in which they are found as a consequence of their low mobility. Although the main interest in terrestrial nematodes originates from the harmful effects some plant parasitic nematodes can exert on agricultural crops, there are many other nematode species contributing to soil fertility by influencing decomposition and mineralization (Anderson *et al.*, 1981; Ingham *et al.*, 1985). Nematodes play a prominent role in terrestrial food webs (De Ruiter *et al.*, 1995).

Nematodes belonging to the terrestrial bacterial feeders inhabit the interstitual water of soil particles (Houx and Aben, 1993). Therefore, they are subjected directly to the dissolved fraction of contaminants in soils, apart from being subjected indirectly via the foodsource. This means that as an indicator of exposure, the biomarker response in these free-living nematodes is predominantly directly related to the bioavailable fraction of pollutants in soils. After extraction from the soil, many of these terrestrial bacterivorous nematodes can easily be reared in the laboratory in growth media or on agar plates with bacteria as foodsource.

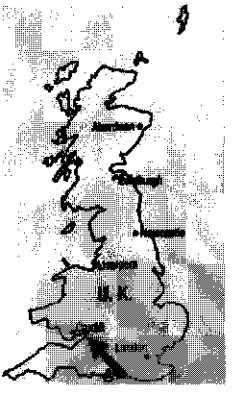
One of the examples of such a free-living terrestrial bacterivorous nematode species, easy to rear in the laboratory, is *Plectus acuminatus* (Nematoda, Torquentia, Plectidae) Bastian 1865. This is a parthenogenetic species, though males do very rarely occur, with an egg-to-egg period of approximately 3 weeks and a life span of about 3 months. It is ubiquitous in the moderate regions of the world and occurs in soils in the Netherlands (Bongers, 1988) and the UK (Arts, unpublished). It is a suitable species for toxicity tests (Kammenga *et al.*, 1996a). This nematode species has already been used in studies focusing on the lethal and sublethal effects of toxicants, including the metals copper and cadmium (Kammenga *et al.*, 1994; Kammenga and Riksen, 1996; Kammenga *et al.*, 1996a and 1996b). So far, there are no reports on hsp responses in this nematode species, except for the ones deriving from this thesis.

Another example of a free-living terrestrial bacterivorous nematode species, easy to rear in the laboratory, is *Caenorhabditis elegans* (Nematoda, Secernentea, Rhabditidae) Maupas 1899, the most investigated nematode species in laboratory experiments that exists. *C. elegans* strain N2 has originally been extracted from soil from the area of Bristol, UK. It has been reared and maintained in the laboratory for decades with *E. coli* as foodsource. *C. elegans* can also be reared axenically in liquid media. It is a self-fertilizing hermafrodite, though males do occur in low frequency. It is homozygous and doesn't suffer from any inbreed depression. Its life cycle is very short with an egg-to-egg period of nearly 3 days and a total life span of about 20 days at 20°C (Wood, 1988). Therefore, this nematode species is very suitable to study life-cycle traits and perform multi-generation experiments.

1.5 The BIOPRINT II project

The main part of the present research was incorporated in the BIOPRINT II project funded by the European Union. The main objective of this project was the validation of biochemical fingerprint techniques for assessing the exposure and effect of toxicants on soil invertebrates in the field (Kammenga and Simonsen, 1997; Kammenga *et al.*, 2000). To achieve this goal, the induction of biomarker responses in several soil invertebrate species of different trophic levels was studied in (i) field populations and (ii) translocated laboratory specimens exposed to polluted soils on site using a range of developed and novel bioassay techniques. The selected polluted field area where all biomarkers were evaluated simultaneously was the Avonmouth area, situated in the UK near the Severn estuary not far from Bristol (see Fig. 3). The Avonmouth area is subject to high levels of aerial metal input, principally from a primary cadmium, lead and zinc smelter (see Fig. 4). This area is very suitable for invertebrate biomarker testing due to the extensive knowlegde of the status of invertebrate populations from past (and present) studies and the presence of suitable field sites. Previous studies of soil contamination in the Avonmouth area have indicated that there is an elevation of metal concentrations in soils at sites up to 15 kilometers downwind of the smelter, with the degree of metal contamination decreasing exponentially with distance from the factory (Spurgeon and Hopkin, 1995 and 1996)(see Fig. 5). The most important pollutants are zinc, cadmium, copper and lead. For the BIOPRINT II project, seven sites were selected along the metal gradient, located in grassland areas and oak stands. For an extensive description of the area, the selected sites, their properties and contaminant concentrations see Filzek *et al.* (in press).

To incorporate a potential suitable nematode biomarker in the suite of biomarkers of the BIOPRINT II project, we first have selected an hsp biomarker in a free-living bacterivorous nematode species upon metal exposure in laboratory experiments. This biomarker in this nematode species should be applicable in metal polluted field situations both in the Netherlands and in the UK. Because *C. elegans* has not been found in soils in the Netherlands yet (Bongers, 1988), *P. acuminatus* has been selected for this purpose. In order to measure the hsp response in a nematode species in field experiments, transplantation of the animals is necessary, because it is impossible to determine nematodes to the species level without killing or at least heavily stressing them. *C. elegans* has not been retrieved after transplantation into the field (see chapter 4), making this species unsuitable for the in situ bio-assays in the field. *C. elegans* has been chosen, however, for the multi-generation experiment, because of its fast generation time. In this experiment, the effect of cadmium on fitness after consecutive generations is studied to assess long-term population level effects imposed by this metal.



← Figure 3: Location of the Avonmouth area in the UK indicated by the black arrow

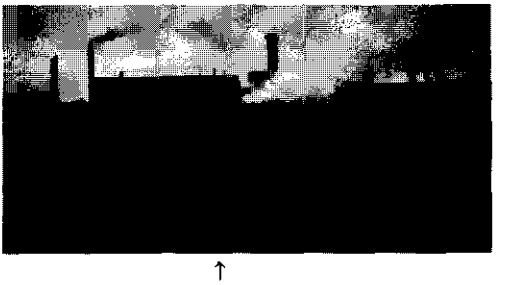


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Figure 4: The pollution source: A primary cadmium, lead and zinc smelter

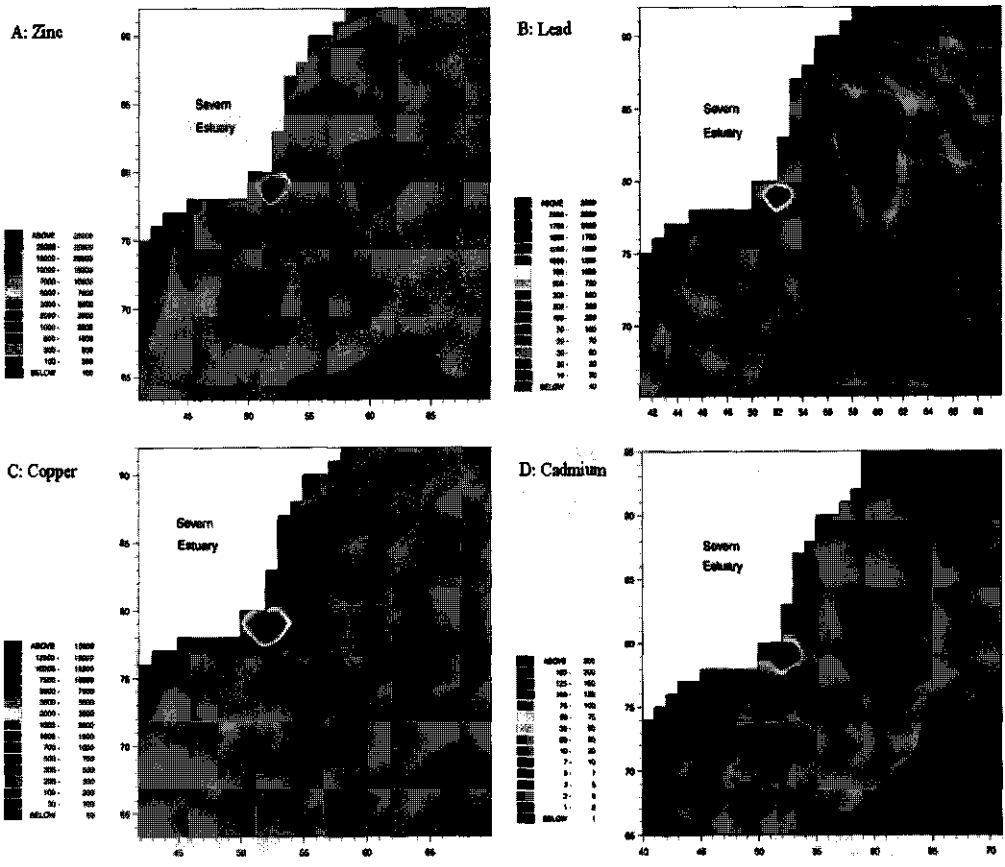

Figure 5: Metal concentrations in surface soils in the Avonmouth area (UK). Metal concentrations are in $\mu g \cdot g^{-1}$ dry weight surface soil, distances in kilometers. A: zinc, B: lead, C: copper, D: cadmium.

Scope of the thesis

The purpose of this thesis is to evaluate metal stress in free-living terrestrial bacterivorous nematodes by measuring the response on the one hand at a very low organisational level (biomarker response) and on the other hand at a high organisational level (fitness consequences at the population level after consecutive generations).

For the biomarker response, first, the most sensitive hsp biomarker for metal exposure in the nematode species *P. acuminatus* is selected in the laboratory. Because both the hsp70 and hsp60 family are generally the most promising as potential biomarkers for monitoring environmental pollution (Sanders, 1990), our attention is focused on analyzing the response of these two hsp families in *P. acuminatus* (chapter 2). In this chapter these responses are determined qualitatively by two dimensional gel electrophoresis and Western blotting. After identifying the potentially most sensitive hsp response to metal exposure and the most suitable antiserum to detect this protein response, in chapter 3 the selected hsp60 response

has been further investigated quantitatively, at the protein level as well as at the mRNA level after exposure to various metals. The mRNA response may be more sensitive and reproducible compared to the protein response and is therefore worth considering. In this chapter, also the hsp60 mRNA sequence and its deduced amino acid translation are elucidated for *P. acuminatus*. In chapter 4, the application of the hsp60 biomarker in *P. acuminatus* is evaluated in an in situ bio-assay in the field experiment along the metal gradient near Avonmouth.

However, the outcome of short-term toxicity studies, such as the hsp responses described in this thesis, may not be used for predicting long-term demographic effects. Because effects at the population level are mediated through effects on fitness, the change in fitness under metal stress in a multi-generation experiment is studied using the nematode *C. elegans* (chapter 5). The strong advantage of testing multiple generations instead of one generation is the detection of possible trade-off mechanisms among life-history traits and fitness consequences, thus eliciting the probable course of the final consequences of chronic metal stress on the existence of the population.

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CHAPTER 2

Hsp60 as a potential biomarker of toxic stress in the nematode *Plectus acuminatus**

ABSTRACT – The induction of heat shock proteins (hsps) in the nematode *Plectus acuminatus* (Nematoda; Plectidae) was studied following exposure to heat, to copper chloride and to cadmium chloride. Mini two-dimensional polyacrylamide gel electrophoresis was used for protein separation. Poly- and monoclonal antibodies raised against hsp70 or hsp60 in various organisms were used to detect the respective hsps by immunoblotting. Both hsp60 and hsp70 could be identified after exposure of the nematodes to heat, indicating the broad cross reactivity among species to the antibodies used. The monoclonal antibody LK-2 was selected for further investigation with the hsp60 response to metals. The induction of hsp60 in *P. acuminatus* was related to increased concentrations of cadmium and copper chloride. For copper chloride, the induction of hsp60 was 3 orders of magnitude more sensitive than was the EC20 for reproduction; for cadmium chloride, the hsp60 induction was 2 orders of magnitude more sensitive. The hsp70 response in *P. acuminatus* was also elevated after exposure of the nematodes to cadmium and copper chloride, but this response was relatively weak compared to the hsp60 response. The results point out that hsp60 induction occurred at concentration levels that are realistic for the field situation. It is therefore suggested that hsp60 may be suitable as a potential biomarker to metal stress in *P. acuminatus*.

*Based on: Kammenga, J.E., Arts, M.S.J. and Oude-Breuil, W.J.M. (1998). HSP60 as a potential biomarker of toxic stress in the nematode *Plectus acuminatus*. *Archives of Environmental Contamination and Toxicology* **34,** 253-258.

2.1 Introduction

Increased attention is paid to the application of heat shock proteins (hsps) as versatile biomarkers to toxicant stress in various organisms. The hsps are a group of proteins within the large family of stress proteins which, apart from hsps, include metal binding proteins, heme oxygenase and glucose related proteins (Sanders, 1990). The hsps are relatively small proteins and can be classified according to their apparent molecular weight of which hsp90 (79-98 kDa), hsp70 (66-78 kDa) and hsp60 (55-65 kDa) are well known representatives (Sanders, 1993). Within the cell, hsps are molecular chaperones that mediate the correct assembly of other polypeptides (Ellis and Van der Vies, 1991). The mitochondrial hsp60 was shown by Martin *et al.* (1992) to form complexes with various polypeptides in organelles exposed to heat stress. They suggested a general mechanism in which hsp binding to a native reductase in the course of denaturation prevented its aggregation and restored the refolding at increased temperatures.

The value of hsps as suitable biomarkers in ecotoxicology was pointed out by various authors and, depending on the species studied, both hsp60 and hsp70 appeared to be useful. Sanders *et al.* (1991) and Cochrane *et al.* (1991) reported on the induction of hsp60 to copper in the blue mussel *Mytilus edulis* and the rotifer *Brachionus plicatilis* respectively. Köhler *et al.* (1992; 1996) found hsp70 induction by heavy metals in the isopod *Oniscus asellus* and the slug *Deroceras reticulatum*. Also Williams *et al.* (1996) reported on the accumulation of hsp70 in gills of rainbow trout exposed to metal-contaminated water.

Few papers have focussed on the use of hsp induction in nematodes as a potential biomarker for toxic stress. Nematodes play a vital role in decomposition processes and are widely abundant in many different soil types. Within ecotoxicology, different nematode species have been used to assess the impact of toxicants on either lethal or sublethal parameters (Donkin and Dusenbery, 1993; Kammenga *et al.*, 1996a; Van Kessel *et al.*, 1989). At present, the nematode *Caenorhabditis elegans* has been used as a model organism for hsp detection after exposure to various stressors. Differential expression of hsp70 to heat and cadmium has been recorded in transgenic strains carrying a *lacZ* reporter gene fused to a hsp promoter sequence (Stringham and Candido, 1994; Guven and De Pomerai, 1995).

To further assess the suitability of the hsp response in nematodes, we investigated the hsp60 and hsp70 induction in *Plectus acuminatus* (Nematoda; Plectidae) Bastian 1865. *P. acuminatus* is a free-living species (i.e. not associated with plant roots or fungi) and is ubiquitous in various soils (De Goede *et al.*, 1993). It feeds on bacteria and reproduces parthenogenetically. *P. acuminatus* has already been used in studies focussing on the sublethal effect of toxicants at the population level and recently a soil toxicity test was developed in standardised soil (Kammenga *et al.*, 1996b).

In this chapter we investigate the suitability of the hsp response as a potential biomarker in *P. acuminatus* exposed to heat stress, copper and cadmium using mini two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and antibody staining. Mini 2D-PAGE has succesfully been used to identify a large number of proteins in plant-parasitic nematodes (De Boer, 1996). The use of 2D-PAGE enabled us to study possible isoforms of the hsps induced. The application of immunological techniques for measuring stress protein induction appears to be very versatile and offers a direct method for studying stress in organisms (Sanders *et al.*, 1994).

2.2 Materials and methods

2.2.1 Nematode culturing

The nematode *P. acuminatus* was originally extracted from the top mineral layer of arable soil at the Binnenhaven in Wageningen, The Netherlands. Stock cultures have been kept in the laboratory for three years and have subsequently been used for supply of complete life-cycle and toxicity experiments. The cultures of nematodes were kept on 0.5 % technical agar mixed with 0.05% proteose pepton and fed with *Acinetobacter johnsonii*, a soil inhabiting bacterium, at a density of 2.10^8 cells/ml. Bacterial densities were measured with a spectrophotometer (Shimadzu, UV-160, 560 nm). To obtain individuals of known age, females taken from the stock culture were allowed to lay eggs for two hours after which the adults were removed. The eggs were then allowed to hatch to obtain a synchronized population.

2.2.2 Hsp detection

Hsp induction was assessed in adult 3-weeks-old females after exposure to heat, to cadmium chloride or to copper chloride in water. The water contained a defined mixture of minerals with concentrations resembling those found in interstitial water of sandy forest soils (Schouten and Van der Brugge, 1989). Heat shock experiments were conducted in water where 110 females were kept at 20°C (control, 24 hours) and 5 or 60 minutes at 37°C. In a separate experiment, 110 females were exposed for 2 hours at 20°C to the following range of metal concentrations: 0.004, 0.04 and 0.4 mg $CuCl_2 \cdot l^{-1}$, and 0.007, 0.07, 0.7 mg $CdCl_2 \cdot l^{-1}$. The highest $CdCl_2$ concentration was 1% of the LC50 (24 hours) in water (Kammenga *et al.*, 1994). This percentage was the same for $CuCl_2$ (the LC50 for copper, however, was determined in a separate experiment).

Mini 2D-PAGE was conducted according to De Boer *et al.* (1992) using the Mini Protean II 2-D Cell system of Bio-Rad Laboratories (Hercules, USA). Iso-electric focussing (IEF) was performed in capillary tubes (length 77 mm, inner diameter 1.1 mm). The IEF gels contained 3.78% (w/v) acrylamide, 0.22% (w/v) bis-acrylamide, 9.15 M urea, 1.6% (w/v) ampholytes pH 5-7, 0.4% (w/v) ampholytes pH 3-10, 0.013% (w/v) ammonium persulphate, and 0.10% (v/v) TEMED. The tubes were filled by capillary action with gel solution. They were then pressed with one side in plasticine and the gel was allowed to polymerize for 1 hour.

Protein samples from a pool of 110 females (approximately 6.6 µg protein according to the method of Bradford (1976)) per gel were prepared as described by Bakker and Bouwman-Smits (1988) and De Boer *et al.* (1992). Protein samples were applied on top of the focussing gel with a Hamilton syringe (type #705, Hamilton Company, Nevada, USA). The remaining space in the tube was filled with a solution containing 5.2 mM Tris-HCl pH 7.4, 8.41 M urea, 0.85% (w/v) ampholytes pH 5-7, and 0.21% (w/v) ampholytes pH 3-10. The cathode buffer contained 20 mM NaOH, the anode buffer 10 mM phosphoric acid. Focussing was accomplished with the following voltage schedule: 16.5 h 10 V, 90 min 180 V, 30 min 270 V and 80 min 603 V using a D.C. power supply (Buchler instruments, nr. 51506, 115 V.A.C.). After focussing, the gels were extruded in an equilibration buffer containing 62.7 mM Tris HCl pH 6.8, 2.3% (w/v) SDS and 7.8% (v/v) glycerol. Further details can be found in De Boer *et al.* (1992).

Second dimension electrophoresis was performed using a Biorad model 1000/500 power supply, 20 min, 10 mA/gel and 60 min 20 mA/gel. The proteins were transferred to PVDF (Poly-Vinylidene Di-Fluoride) membrane and blotted for 60 min (0.8 mA/cm2 gel, maximum

voltage) at 20°C in 48 mM Tris, 39 mM glycine in 100 ml methanol and 400 ml bidest. Subsequent blocking occurred in PBS buffer + 0.1% Tween 20 + 5% skimmed milkpowder. After removal of the blocking solution with PBS buffer + 0.1% Tween 20 for 2 x 5 min, primary antiserum (1:1000 dilution) in PBS buffer + 0.1% Tween 20 + 1% skimmed milk powder was added and left for 2 hours. After washing with PBS buffer + 0.1% Tween 20 for 3 x 5 min incubation of the second antibody (alkaline phosphatase-conjugated rat anti-mouse (dilution 1:5000) or anti-rabbit (1:1000)) took place for 1 hour. The membrane was washed with water 5 times for 5 min and transferred to substrate buffer (0.1 M ethanolamine-HCl (pH 9.6) + 4mM $MgCl_2$) to allow for substrate binding for approximately 2 hours. Subsequently, the membranes were washed with distilled water and air dried.

As described, following 2D-PAGE, hsp induction was revealed by subsequent Western blotting employing mono- and polyclonal antibodies. The following six anti hsp antibodies raised in mouse or rabbit (Stressgen, Biotechnologies corp., Victoria, BC, Canada or others as indicated) were used. Monoclonals: mouse anti-human hsp60 (clone LK-2), mouse anti-bovine hsp70 (clone BRM-22, obtained from Sigma), mouse anti-human hsp70 (clone C92F3A-5), anti guinea pig hsc70 (directed against the constitutive form of hsp70, clone 1B5). Polyclonals: rabbit anti-moth (*Heliothus virescens*) hsp60, rabbit anti-cyanobacterium (*Synechococcus sp.*, strain PCC7942) GroEl, which is equivalent to hsp60. For all data, three replicates were examined.

The following molecular weight markers were used: phophorylase B (112 kDa), bovine serum albumin (84 kDa), ovalbumin (53.2 kDa), carbonic anhydrase (34.9 kDa), soybean trypsin inhibitor (28.7 kDa) and lysozym (20.5 kDa). The markers were run in one lane of the second dimension.

2.3 Results

Exposure of *P. acuminatus* to a heat shock of 37°C resulted in the induction of various protein spots. Figure 1 (A-H) shows the immunoblots for various antibodies. The central area of each blot is shown and numbers were assigned to the major spots.

Mouse anti-bovine hsp70 (clone BRM-22) staining resulted in one small spot (1) at approximately 75 kDa and two small spots (2, 3) at about 73 kDa in the control treated animals after 24 hours of exposure at 20°C (Fig. 1A). The spots could be assigned to be different members and isoforms of the hsp70 family. Spot x is regarded as an artefact due to a small air bubble in the gel. Protein spots 1, 2 and 3 were also identified after 5 minutes of exposure to 37°C, only expression was more pronounced (Fig. 1B). In addition, a new weak spot (4) appeared at about 73 kDa and two new spots were found at about 40 kDa (5, 6). After 60 minutes of exposure, the spot pattern was not more pronounced (Fig. 1C). Both exposure periods at 37°C resulted in a strong induction compared to the control at 20°C.

Staining with mouse anti-human hsp60 (clone LK-2) in control females resulted in one small protein spot (7) at about 58 kDa, which could be designated to the hsp60 family, and one weak spot (8) at 35 kDa (Fig. 1D). Two new spots (9, 10) were found at about 60 kDa and one spot at 58 kDa (7) after 5 minutes of exposure to 37°C. A fourth weak spot (8) was found at approximately 35 kDa (Fig. 1E). After 60 minutes, induction was more pronounced (Fig. 1F), the spots 9 and 10 overlapped and the spot at 35 kDa became more pronounced. Both exposure periods at 37°C resulted in a strong induction compared to the control.

Rabbit anti-moth hsp60 polyclonal showed a strong increase in protein spot pattern after 60 minutes at 37°C (Fig. 1H) compared to 24 hours at 20°C (Fig. 1G). Spots 11-15 had a weight of about 54 kDa, 16 was 59 kDa and all could be assigned to be members of the hsp60

family. All other weak spots were caused by non-specific binding to other proteins because of the polyclonal being less specific than a monoclonal antibody. Spot 17 was 44 kDa and 18 (Fig. 1H) was about 35 kDa.

All other antibodies tested (mouse anti-human hsp70 (clone C92F3A-5), anti-guinea pig hsc70 (clone 1B5), and the polyclonal rabbit anti-cyanobacterium (strain PCC7942) showed coss reactivity with various proteins in the nematode, however, there was no consistent relationship with increased temperature exposure.

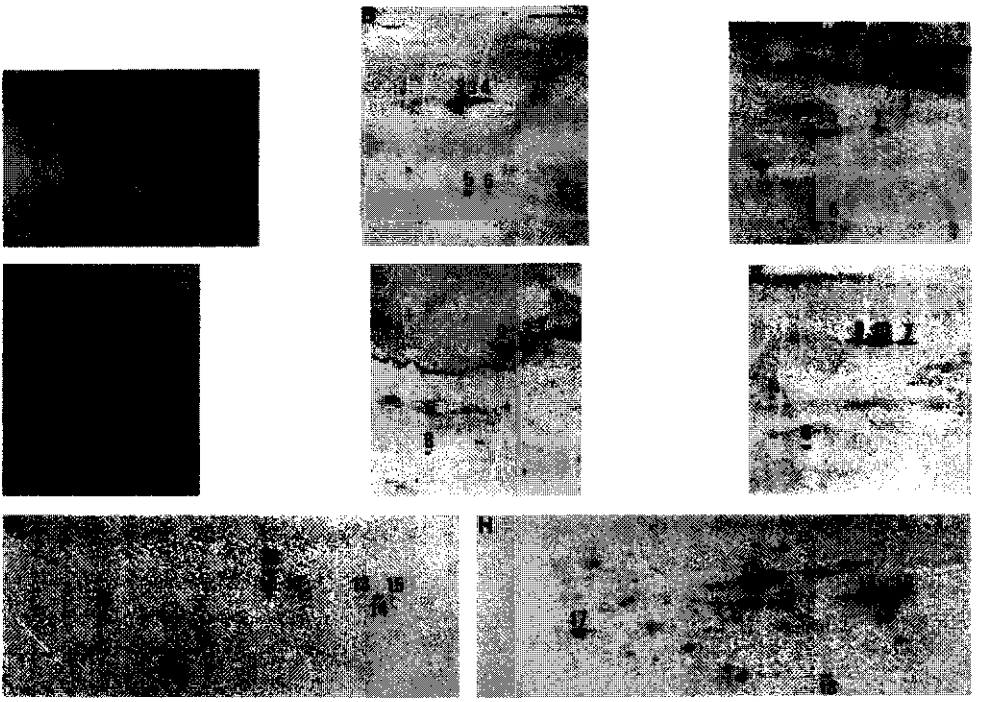

Figure 1: Two dimensional immunoblots of pooled 110 adult *P. acuminatus* females exposed for 5 and 60 min to 37°C and 24 h to 20°C (control) in water. Mouse anti-bovine hsp70 (clone BRM-22), control (A), 5 min (B), 60 min (C); mouse anti-human hsp60 (clone LK-2), control (D), 5 min (E), 60 min (F); rabbit anti-moth hsp60 polyclonal, control (G), 60 min (H).

Since the use of both mouse anti-human hsp60 (clone LK-2) and mouse anti-bovine hsp70 (clone BRM-22) appeared to be successful in revealing hsp60 and hsp70 induction respectively in *P. acuminatus* to heat stress, these antibodies were selected for further investigation in a biomarker applicability study for metal stress using 2D-PAGE and antibody staining. Figure 2 shows the two dimensional immunoblots of metal-induced hsp60. Numbers 1-3 and 4-6 refer to increasing concentrations of copper and cadmium chloride, respectively. In the control (CO), a weak spot was identified. This spot was weaker than the one shown in the control after 24 hours (Fig. 1D). Exposure to the metals resulted in hsp60 induction compared to the control and the induction increased at higher exposure concentrations. Two distinct spots could be identified of the same weight but with different grey density. Both spots resembled spots 9 and 10 which have been found after the heat shock treatment (Fig. 1E, F).

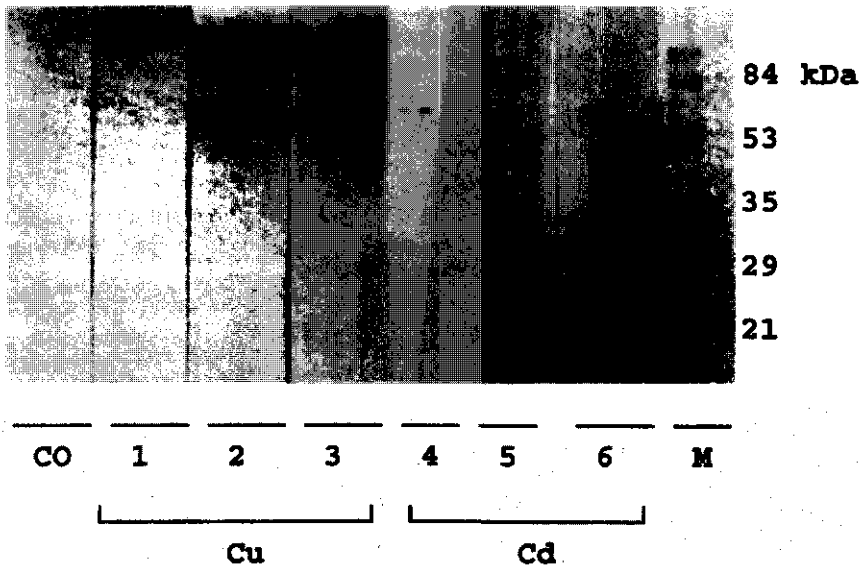

Figure 2: Induction of hsp60 as revealed by using two dimensional immunoblotting in the nematode *P. acuminatus* after 2 hours of exposure to cadmium and copper chloride in water. M=molecular weight markers (kDa). CO=control, 1=0.004, 2=0.04, 3=0.4 mg $CuCl_2 \cdot l^{-1}$; 4=0.007, 5=0.07, 6=0.7 mg $CdCl_2 \cdot l^{-1}$. Protein samples are from a mixture homogenate of 110 pooled females.

After 2 hours of exposure to the metals, no apparent hsp70 induction was found as evidenced from immunoblots. It was therefore decided to increase the exposure period up to 24 hours at 20°C at the following relatively high concentration levels: 17.9 mg $CdCl_2 \cdot l^{-1}$ and 1.8 mg $CuCl_2 \cdot l^{-1}$. Figure 3 shows the two dimensional immunoblots of metal-induced hsp70 proteins using the same numbers for spots as in Figure 1 A-C. Exposure to cadmium chloride (Fig. 3A) and copper chloride (Fig. 3B) resulted in a doublet spot (1) at approximately 75 kDa and two small spots (2, 3) at about 73 kDa which were more pronounced compared to the control (Fig. 1A). The spot with the letter y is an artefact. Compared to the hsp60 induction, the response of hsp70 was relatively weak even after 24 hours of exposure.

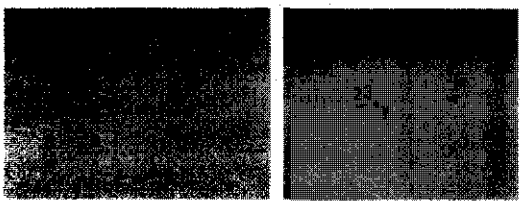

Figure 3: Induction of hsp70 as revealed by using two dimensional immunoblotting in the *nematode P. acuminatus* after 24 hours of exposure to cadmium and copper chloride in water. A: 17.9 mg $CdCl_2 \cdot l^{-1}$, B: 1.8 mg $CuCl_2 \cdot l^{-1}$. Protein samples are from a mixture homogenate of 110 pooled females.

2.4 Discussion

Proteins belonging to the family of hsp60 were shown to be promising biomarkers for stress in the nematode *P. acuminatus* after exposure to heat, to cadmium chloride and to copper chloride. The total amount of protein used (nearly 7 µg per gel) resulted in clear immunoblots. This is in agreement with the findings of De Boer (1996) for other nematode species and different proteins using both silver staining and immunoblotting. Larger amounts of protein resulted in streaking of proteins and led to difficulties in discriminating between different protein spots.

The hsp60 monoclonal antibody LK-2 was successfully used for hsp60 detection which agrees with previous findings on the broad cross-reactivity of this antibody with hsp60 in many different prokaryotic and eukaryotic species (Boog *et al.*, 1992). This may be explained by the fact that the antigenic similarity among members of the hsp60 family is highly conserved (McMullin and Hallberg, 1988). For instance, the epitope recognized by LK-2 is located between the amino acid residues 383-419 of the human hsp60 sequence and corresponds to residues 356-393 of a mycobacterial hsp60 sequence (Boog *et al.*, 1992).

The differences in spot patterns of hsp60 found in the 2D-PAGE immunoblots between control and stressed animals possibly suggests the existence of different subunits. In eukaryotic animals, the hsp60 proteins are found in mitochondria and are arranged in complexes which consist of different subunits of approximately 60 kDa each (McMullin and Hallberg, 1987). These subunits may differ in their iso-electric point leading to a distinct separation pattern of isoforms in the first dimension of the 2D-PAGE. As pointed out by Guven and De Pomerai (1995) for hsp70, the various protein spots within the same weight class may not neccessarily correspond to separate gene products. Some of the diversity shown may have resulted from post-translational processing or even degradation, which may be the reason why we have found a spot at 35 kDa. In agreement with the findings for hsp70 in the nematode *C. elegans* (Guven and De Pomerai, 1995), we have found newly synthesized distinct protein spots within the hsp60 family after exposure to heat or to metals.

Our results can be compared with a number of papers which have focussed on the use of hsps as biomarkers for toxicant stress in both aquatic and terrestrial animals. Most studies dealt with induction of hsp70 proteins. Köhler *et al.* (1992) reported on the response of hsp70 in soil invertebrates exposed in the laboratory to heavy metals. In addition, they found a marked increase in hsp70 induction in isopods taken directly from a heavy metal contaminated site.

The monoclonal antibody BRM-22 raised against bovine cognate stress 70 cross-reacts with a 78 and 72 kDa protein in non-mammals (Sanders *et al.*, 1994). Guven and De Pomerai (1995) also used this antibody in a study on differential expression of hsp70 proteins in response to heat and cadmium in the nematode *C. elegans*. They found an increase in hsp70 after exposure to 10 mg $CdCl_2 \cdot l^{-1}$ for 7 hours at 31°C. Two 73 kDa spots and one doublet spot (which they numbered 11, 12 and 8 respectively, their figure 3) are comparable to the spots we found (nr. 2, 3 and 1). Their immunoblots of cadmium-exposed animals show more protein spots compared to ours, which might be explained by the relatively high cadmium concentration (a difference of a factor 10). Also the exposure period used in the heat shock experiment was 7 hours at 34°C compared to 1 hour at 37° in our studies.

Sanders *et al.* (1991) used Western blot analysis in 1D-PAGE and the polyclonal rabbit anti-moth (*Heliothus virescens*) hsp60 to detect hsp60 induction in the blue mussel *Mytilus edulis* after exposure to copper. The results indicated that hsp60 accumulation was larger in tissues exposed to elevated copper concentrations than in the control. A significant correlation was found between hsp60 accumulation and ambient copper concentrations. In

contrast to our findings, Sanders *et al.* (1991) did not find any background level of hsp60 in the mussel tissue. Many stress proteins, including members of the hsp60 family, however, are constitutively expressed and fulfill essential functions as molecular chaperones (Martin *et al.*, 1992) under normal ambient conditions. Besides this, it could very well be that the nematodes suffered from handling stress, since they were transferred from their laboratory habitat (agar) into water. Using the same polyclonal antibody and 1D-PAGE, Cochrane *et al.* (1991) found a marked increase of stress protein 58, a member of the hsp60 family, in the rotifer *Brachionus plicatilis* exposed to copper at comparable concentration levels such as we used.

The 2D-PAGE system has the advantage over 1D-PAGE in that protein pattern details like isoforms are revealed which will not become visible by using 1D-PAGE. An important prerequisite for a biomarker to be used in regular risk assessment protocols is its rapid and easy deployment. For rapid screening of hsp induction however, the use of 2D-PAGE as a routine analysis can be questioned due to the time-consuming and tedious labour required in processing the second dimension. At present gels are available from various companies with pre-fixed pH gradients which greatly overcome these problems. Since we have found that hsp60 might be a potential biomarker for chemical stress in *P. acuminatus,* 1D-PAGE can be recommended when combined with specific antibody staining using anti-hsp60 in the Western blot.

In general, biomarkers should act as early warnings to chemical stress, meaning that the induction should already become evident far below the concentration level where other sublethal effects become apparent. For copper the EC20 for reproduction in *P. acuminatus* was 6.43 mg $CuCl_2 \cdot l^{-1}$ (Kammenga and Riksen, 1996). We found that hsp60 induction was 1.600 (6.43/0.004) times more sensitive than reproduction. For cadmium the EC20 for reproduction was 0.81 mg $CdCl_2 \cdot l^{-1}$ (Kammenga *et al.*, 1996a), while the induction of hsp60 was 115 times (0.81/0.007) more sensitive. In the mussel *M. edulis*, the hsp60 induction was 10 times more sensitive than adverse effects on biomass production (Sanders *et al.*, 1991). The great sensitivity of 4 μg $CuCl_2 \cdot l^{-1}$ raises questions as to how relevant these hsp60 effect levels are in relation to natural copper concentrations. In ground water, the average concentration for loamy sand is 4.8 μg $Cu \cdot l^{-1}$ and clay 2.0 $\mu g \cdot l^{-1}$ (Slooff *et al.*, 1987). Hence, the minimal levels of copper resulting in hsp60 induction in our study are in comparison with natural background levels. From these findings it can be concluded that hsp60 induction seems to be a remarkably sensitive early warning following minimal levels of chemical stress.

Ideally, a biomarker should be indicative for a specific kind of stressor, i.e. it should be able to discriminate beween different stress factors. Hsps have the major drawback that they are synthesized within the frame of a general response to ambient and internal stress such as heat, food condition and toxicants. Most probably, these different stressors each exert a specific response which could be quantified. For instance, we found that exposure to heat resulted in some different isoforms of hsp60 than exposure to cadmium or copper chloride (compare Fig. 1E and 1F with Fig. 2). The induction of hsps using immunoblot techniques allows for quantification using digital image analysis such as performed by Sanders *et al.* (1991). Before hsps can be used as biomarkers in field assessment programmes, the effect of confounding factors such as temperature and pH should be well known. A different but also important prerequisite for a potential biomarker is the transiency of the biomarker response.

We have shown that hsp60 has potential as a biomarker for chemical stress in the nematode *P. acuminatus*. Immunological methods enhance the sensitivity of direct measurements of stress-induced hsp60 levels. The experiments were all performed on animals which were exposed in water in the laboratory under controlled conditions. The final aim is to focus on hsp60 induction in nematodes exposed in the field using in-situ bioassays.

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CHAPTER 3

Identification of hsp60 in the nematode *Plectus acuminatus* and its quantitative response to metal exposure *

ABSTRACT - The last decade, heat shock proteins (hsps) have provoked the interest of the environmental sciences because they have potential as biomarkers for environmental pollution. The hsp60 response in the terrestrial bacterivorous nematode *Plectus acuminatus* was demonstrated to be a promising biomarker for metal stress (see chapter 2 of the present thesis). This was only measured qualitatively in laboratory experiments. In the present chapter, we aimed to establish a quantitative relationship between the hsp60 response and increasing metal concentrations in laboratory tests. We measured the hsp60 response in *P. acuminatus* at both the protein and mRNA level after 24 hours of exposure to either zinc chloride (0-550 μM) or copper chloride (0-59 μM), the protein response also after 24 hours of exposure to cadmium chloride (0-109 μM). Furthermore, we identified hsp60 in *P. acuminatus* by elucidating its full-length mRNA sequence and deduced amino acid translation and comparing this to other known sequences.

After exposure of the nematodes to zinc chloride, a significant optimum curve was found for the hsp60 response at the protein level, with a maximum induction of over 8 fold the control response at a concentration of 291 μM zinc chloride. A significant hsp60 increase at the protein level was also detected with increasing copper chloride concentrations, but the maximum hsp60 induction was not reached within the investigated copper concentration range. When the nematodes were exposed to cadmium chloride, no significant trend was observed. At the mRNA level, in *P. acuminatus* no considerable hsp60 induction was obtained when compared to control levels and to the protein levels at the investigated metal concentration range after 24 hours of exposure. Though the variability at the hsp60 protein level in *P. acuminatus* was much higher compared to the hsp60 mRNA level, the increase upon metal exposure was much higher at the protein level and occurred at higher metal concentrations. Therefore, the hsp60 protein response in *P. acuminatus* may have more potential as a biomarker for metal stress than the hsp60 mRNA response.

* Based on: Arts, M.S.J., Stürzenbaum, S.R. and Kammenga, J.E. Identification of hsp60 in the nematode *Plectus acuminatus* and its quantitative response to metal exposure (submitted).

3.1 Introduction

The cellular stress response is the mechanism of the cell to maintain vital cellular functions, also in situations deviating from normal conditions and provoking stress. Part of this stress response is the heat shock protein (hsp) response, first discovered upon heat exposure but later found to be induced by a wide variety of chemical, physical and biological stressors (e.g. listed in Nover, 1991 and Sanders, 1993). Following exposure to a stressor, cellular gene expression is altered resulting in a cessation of normally occurring protein synthesis and the induction or augmentation of hsp synthesis. Hsps possibly all function as molecular chaperones (Ellis, 1987), for one, facilitating the synthesis, folding, assembly and intracellular transport of many proteins, reducing protein denaturation and aggregation and aiding in protein renaturation (e.g. Ellis and van der Vies, 1991; Parsell and Lindquist, 1993). Each hsp is the member of a multigene family, regulated by different promotors and coding for closely related protein isoforms (Lindquist, 1986). Based on their molecular weight, hsps can be classified into different families (Sanders, 1993).

The family of 55-65 kDa is called chaperonin and its members have thus far been found in eubacteria and in eukaryotic cells, almost exclusively in organelles which are probably of endosymbiotic origin (mitochondria, chloroplasts) (Hemmingsen *et al.*, 1988) designated hsp60, stress-60, cpn60, GroEL (*E. coli*) or RuSBP (Rubisco Subunit Binding Protein (chloroplast)). Furthermore, Tcp-1 (t-complex polypeptide 1), a protein family distantly related to hsp60 and located in the cytosol of eukaryotic cells and in archaebacteria, is sometimes also referred to as a member of the chaperonin family (Gupta, 1990; Trent *et al.*, 1991).

Chaperonins mediate the correct folding of polypeptide folding intermediates and their assembly with other protein components into oligomeric structures (Welch, 1993). They are also involved in secretion and (membrane) translocation of a number of protein precursors and aid damaged and misfolded proteins to refold correctly to their native conformation or otherwise, making them sensitive to proteolytic digestion (Gething and Sambrook, 1992; Parsell and Lindquist, 1993). More information about e.g. the structure of chaperonins can be found in chapter 1 of the present thesis.

Hsp60 is a nucleus-encoded, constitutively expressed protein. Under stressfull conditions, the hsp60 expression can be dramatically increased. Although most publications are in the medical field, the last decade chaperonins and other hsps have provoked the interest of the environmental sciences because of their potential as biomarkers for environmental pollution (Sanders, 1990). Most hsp60 research in this perspective has been focused on the aquatic environment, though the last years also attention has been paid to terrestrial invertebrates.

Nematodes are the most dominant group of multicellular organisms on earth and are abundant in all habitats and feeding types. Bacterivorous nematodes can be extracted from the soil efficiently and be reared in the laboratory. They play a major role in decomposition processes of organic matter by stimulating N-mineralization and nutrient cycling (Anderson *et al.*, 1981). The soil-inhabiting bacterivorous nematode *Plectus acuminatus* (Nematoda, Plectidae) Bastian 1865 is widely abundant in the moderate regions of the world. In toxicity tests, the effect levels of several contaminants on this species were comparable to similar tests using other invertebrates (Kammenga *et al.*, 1996a). To monitor proteotoxic metal effects in this nematode, hsp60 appeared to be more upregulated in response to increasing cadmium en copper chloride concentrations compared to hsp70 (Kammenga *et al.*, 1998). Because the hsp60 response in *P. acuminatus* has the potential to be a suitable biomarker for metal stress (chapter 2 of this thesis), we studied this response in more detail.

In the present chapter we identified and characterized hsp60 in the nematode *P. acuminatus* and measured its response at the mRNA and protein level after exposure to several metals in laboratory experiments. The activation of a set of *hsp* genes results in a rapid and transient increase in transcription, the accumulation of hsp mRNAs and the synthesis of hsp (Bienz and Pelham, 1987). Because the hsp60 response at both the mRNA and protein level are probably transiently increased, both are interesting to analyze in order to select the one that has the best potential as a biomarker for metal pollution. Both responses were measured quantitatively. The protein response was measured by using one dimensional polyacrylamide gel electrophoresis and subsequent Western immunoblotting, after which the grey values of the protein bands were measured by densitometric image analysis. The mRNA response was determined by reverse transcription followed by fluorescent based quantitative real-time polymerase chain reaction. Using these methods, the mRNA response could more adequately be quantified compared to the protein response and therefore more accurately be tested.

3.2 Materials and methods

3.2.1 Culturing

The nematode *P. acuminatus* was originally extracted from the top mineral layer of arable soil at the Binnenhaven in Wageningen, The Netherlands, in 1993. Stock cultures have been kept in the laboratory on 1% or 3% technical agar (Oxoid Limited) mixed with 0.05% proteose pepton (Difco Laboratories) in plastic petri dishes (6 cm diameter, Greiner) at 20°C. The nematodes were fed with *Acinetobacter johnsonii* strain 210A (the Netherlands Culture Collection of Microorganisms, access number LMAU A130 (Bonting *et al.*, 1992)). *A. johnsonii* was cultured in sterile yeast extract (Difco Laboratories, 4 gr/l) in aerated bottles at 28°C overnight until a concentration of approximately $2 \cdot 10^8$ cells per ml was obtained and kept at 4°C until use. Prior to adding the nematodes to the plates, 30 µl of bacteria were pipetted on the plates, spread with a glass rod and allowed to grow overnight at 28°C. From one individual of the nematode stock cultures a population was reared for the use in all the experiments.

3.2.2 Sample preparation

The nematodes were rinsed off the plates over a 10 µm sieve (40 mm diameter, Laboratory of Nematology, Wageningen) with standard pore water (SPW). SPW is an aqueous solution of a defined mixture of minerals, resembling the interstitial water of sandy forest soils (Schouten and Van der Brugge, 1989). After collecting the nematodes in 10 ml plastic tubes (Greiner) and centrifuging them for 2 minutes at 760 g (MSE super minor centrifuge), the overlaying water was removed and the nematodes were collected in 1.5 ml eppendorf tubes. The nematodes were spun (16,000 g, Eppendorf Centrifuge 5415 C) and the overlaying water was removed until the desired volume was reached.

Metal stock solutions were pipetted in liquid 1% technical agar with 0.05% proteose pepton (1% PPA) and mixed, thus obtaining the following final concentrations: 0, 110.1, 165.1, 220.1, 275.2, 330.2, 440.3, 550.3 µM zinc chloride ($ZnCl_2$ (Merck)), 0, 11.7, 23.5, 35.2, 46.9, 58.7 µM copper chloride ($CuCl_2 \cdot 2H_20$ (Sigma)), and 0, 10.9, 27.3, 54.6, 109.1 µM cadmium chloride ($CdCl_2$ (Merck)) respectively. The agar-metal mixtures were poured into plastic petri dishes (6 cm diameter, Greiner).

30 µl of *A. johnsonii* (approximately $6 \cdot 10^6$ cells) were pipetted upon the solidified agar and spread with a glass rod, after which the concentrated nematodes were pipetted on top of this and spread with a glass rod too. The plates were sealed with parafilm and incubated at 20°C for 24 hours. Subsequently, the nematodes were rinsed off the plates and concentrated as described until the final volume of each sample weighted 20 mg. The samples were frozen in liquid nitrogen and kept at -80°C until further use.

3.2.3 Isolation of mRNA

mRNA was isolated from 150-500 (RT-PCR) or 7000 (cDNA library) nematodes per sample using the Dynabeads® mRNA DIRECT kit (DYNAL A.S., Skøyen, Norway). The small scale direct isolation of mRNA from solid animal tissues was applied according to the manufacturers recommendations. All reagents used were supplied in the kit. All materials (except for the samples) were RNAse free. The frozen nematode samples were transferred into small glass mortars (23x14 mm, with a 5x7 mm bulge at the bottom (Laboratory of Nematology, Wageningen)) at 4-7°C. After adding 20 µl of Lysis/binding buffer, the samples were ground with a pestle fitting in the bulge by using a fixed drill (Heidolph Typ 50113 RZR 3) with the pestle as bit at 200 turns/min at 4-7°C. After 4 minutes of grinding, another 20 µl of Lysis/binding buffer were added and grinding for another minute took place. The ground samples were transferred into a 0.5 ml eppendorf tube and put on ice. The pestle and the glass mortar were flushed with 100 µl of Lysis/binding buffer altogether, after which the liquid was added to the samples. The samples were centrifuged at 16,000 g at 4-7°C for 30-60 sec (Eppendorf Centrifuge 5415 C) and put on ice. The manufacturers protocol for direct mRNA isolation from crude lysate was performed with the use of 200 and 150 µl of Washing buffer with LiDS, 150 and 75 µl of Washing buffer (without LiDS) and 20 µl of cDNA wash mixture. The mRNA was eluted from the Dynabeads Oligo $(dT)_{25}$ in 11 µl of Elution solution according to the manufacturers protocol.

3.2.4 Construction and identification of full-length cDNA

An adapter ligated rapid amplification of cDNA ends (RACE) ready cDNA mini-library was constructed using the Marathon™ cDNA Amplification Kit (CLONTECH Laboratories, Palo Alto, USA) according to the manufacturers recommendations. Gene-specific primers were designed to amplify the 5' and 3' end of *P. acuminatus* hsp60 cDNA: 5'-CTTGTAGACCGACCTTGAGCCTGT-3' for the 5'-RACE reaction and 5'-ACAGGCTCAAGGTCGGTCTACAAG-3' for the 3'-RACE reaction respectively. Both these gene-specific primers were reverse and complementary to each other, because this primer region was the only sequence region (*P. acuminatus hsp60* gene (partial), EMBL/GenBank Libraries accession number AJ130947) not to include self priming and having a high Tm, thus enabling touchdown PCR. All primers were purified by HPSF technology. The touchdown PCR was performed in a DNA Thermal Cycler 480 (Perkin Elmer) using the following programme: 94°C for 1 min; 5 cycles: 94°C for 30 sec, 72°C for 2 min; 5 cycles: 94°C for 30 sec, 70°C for 2 min; 23 cycles: 94°C for 20 sec, 68°C for 2 min. The PCR products were separated on a 1% agarose gel with 0,005% Ethidium Bromide (Gibco BRL). Appropriate bands were purified using the DNA extraction kit (Hybaid) with a final elution volume of 14 µl of which 7 µl were directly used to ligate into the pGEM-T vector (Promega) according to the manufacturers recommendations. Positive clones were sequenced using the PCR based Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Buckinghamshire, UK). The resulting

amplified DNA fragments were separated on a gel matrix using a LI-COR DNA sequencer model 4000L (Li-cor, Lincoln, NE, USA) and analyzed with the BASE ImagIR Image Analysis data collection package (Version: 02.21) (Li-cor, Lincoln, NE, USA).
To confirm the nucleotide sequence of the primer defined region, a further PCR was performed in composing the initial predefined primer area by using the following primers: 5'-GGATGTTGTGCCCGCTTTGGA-3' and 5'-TTCGCTGCCACCGCCAACCT-3'. The resulting band was extracted, cloned and sequenced as described before.

The full-length *P. acuminatus* hsp60 sequence was determined on both strands of the 5' fragment, the 3' fragment and the 5'-3' overlap region. The resulting sequence was compared to all database entries searching for nucleotide and amino acid homologies (BLASTN and BLASTP searches respectively (Altschul *et al.*, 1990; Gish and States, 1993) and submitted to the EMBL/GenBank Libraries (accession number AJ130877).

To quantify the hsp60 response in *P. acuminatus*, an invariant standard was required. A conserved, housekeeping gene which is often used as a reference is actin (e.g. Stürzenbaum *et al.*, 1998; Dumoulin *et al.*, 2000). Therefore, primers specific for actin were designed based on conserved regions of known actin sequences from various organisms as extracted from the EMBL/GenBank Libraries: 5'-GGAGCAATGATCTTGATCTT-3' to amplify the 5' end and 5'-TGGCA(CT)CACACCTTCTACAA-3' to amplify the 3' end. These actin primers were applied to amplify *P. acuminatus* actin cDNA using the adapter ligated RACE ready cDNA mini-library of *P. acuminatus* as described. The resulting products were cloned and sequenced as described. The full-length *P. acuminatus* actin sequence was determined on both strands of the 5' and 3' product. It was compared to all database entries searching for nucleotide and amino acid homologies (BLASTN and BLASTP searches respectively (Altschul *et al.*,1990; Gish and States, 1993) and submitted to the EMBL/GenBank Libraries (accession number AJ012665).

3.2.5 1D-SDS-PAGE and Western blotting

The hsp60 response at the protein level was determined by using one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) (Laemmli, 1970) and subsequent Western blotting (Towbin *et al.*, 1979). The frozen nematode samples were transferred into small glass mortars (23x14 mm) with a bulge (5x7 mm) at the bottom. After adding 20 µl of extraction buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 2% DTT, 2mM Pefabloc SC (Merck)), the samples were ground with a pestle fitting in the bulge by using a fixed drill (Heidolph Typ 50113 RZR 3) with the pestle as bit at 200 U/min. The samples were repeatedly ground for 20 seconds and cooled for 10 seconds on ice until 6 minutes had passed. The ground samples were transferred into eppendorf tubes. 20 µl of extraction buffer were used to flush the glass mortars and were then added to the samples. Subsequently, the samples were centrifuged at 16,000 g for 5 minutes (Eppendorf Centrifuge 5415 C). The supernatants were transferred into eppendorf tubes, boiled for 5 minutes, cooled on ice for at least 1 minute, frozen in liquid nitrogen and kept in the -80°C until analysis. Analysis was performed as described by De Boer *et al.* (1996) and Kammenga *et al.* (1998). The monoclonal antibodies mouse anti-human hsp60 clone LK-2 (cat. no. SPA-807, StressGen Biotechnologies Corp., Victoria, Canada) (1:1000 diluted) and mouse anti-chicken actin clone C4 (ICN Biomedicals Inc., Aurora, Ohio, USA) (1:400 diluted) were applied. However, the actin response was so hugh that it interfered with the hsp60 detectability and therefore was not suitable as an internal standard in the protein experiments. As a consequence, instead of actin tropomyosin, a protein of the striated muscle filaments, was used as an internal standard in the protein assays. The monoclonal mouse

anti-*Globodera rostochiensis*, a plant parasitic nematode, tropomyosin antibodies (De Boer *et al.*, 1996) were applied (1:1000 diluted). As secondary antibodies alkaline phosphatase-conjugated rat anti-mouse antibodies (Jackson ImmunoResearch laboratories, West Grove, Pennsylvania, USA) were employed (1:5000 diluted). Grey values of the blot bands were measured by densitometric image analysis (Magiscan image analysis system (APPLIED IMAGING, Tyne & Wear, UK) Programme Genias). The data were processed using the computerprogramme MIDAS (APPLIED IMAGING, Tyne & Wear, UK) and the hsp60 per tropomyosin value (h/t value) was calculated for each sample. Although analyses comprise hsp60 of bacteria associated with the nematodes by adhesion to the cuticula or by ingestion by the nematodes, the respective stress protein level can be assigned to the nematodes themselves due to the comparatively small mass of the bacteria.

3.2.6 RT real-time PCR

The hsp60 response at the mRNA level was determined by using reverse transcription followed by a quantitative real-time polymerase chain reaction. First mRNA (see paragraph 3.2.3) was reverse transcribed using the SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (Life Technologies). First strand synthesis was performed with $Oligo(dT)_{12\text{-}18}$ priming according to the manufacturers protocol using only reagents supplied in the kit.

The cDNA was amplified by fluorescent based quantitative PCR using a LightCycler (Idaho Technology, Idaho Falls, USA) as described by Wittwer *et al.* (1997). Conditions were set and optimized as described by Stürzenbaum *et al.* (1998) and Stürzenbaum (1999). Primers specific for *P. acuminatus* actin were designed to amplify a fragment of 294 bp: 5'-TCCAAGGCGACATAGCAGAG-3' to amplify the 5' end and 5'-CGAGACCTTCAACACGCCTG-3' to amplify the 3' end. For hsp60 amplification the primers used to confirm the nucleotide sequence of the primer defined region (see paragraph 3.2.4) were applied. Each PCR reaction contained 0.3 pM of each primer (synthesized by Life Technologies), 0.2 mM dNTPs (Promega), 0.1 unit per µl of *Taq* polymerase (Promega), PCR buffer ((Biogene), final concentration 50 mM Tris, 250 µg/ml BSA, 4 mM $MgCl_2$), 5 pM tRNA (*Eschericia coli*, Strain W; Sigma, St. Louis, MO, USA) in order to reduce the formation of primer artefacts (Stürzenbaum, 1999) and 1:15000 dilution of SYBR® Green I (FMC BioProducts, Rockland, ME, USA) which is the fluorescent label. 20 µl of amplification reaction consisted of 18 µl reaction mixture and 2 µl of cDNA (1:10) (plasmid cloned DNA preparation for calibration standards (1 ng – 100 fg), reverse transcribed mRNA for unknown concentrations). The PCR reactions were performed in triplicate in microvolume capillaries (Biogene), 5 µl per capillary, and subjected to the following programme: 40 cycles: 95°C for 1 sec, 62°C for 3 sec, 72°C for 7 sec. Every PCR product was assessed for its amplification specificity by real time melting curve analysis (Ririe *et al.*, 1997), ensuring that only true amplification products were included in the quantification.

3.2.7 Statistics

Based on earlier research, we expected the hsp60 response in *P. acuminatus* to increase upon exposure to increasing metal concentrations (Kammenga *et al.*, 1998) until a maximum response level had been reached. When metal concentrations further increased, we expected the hsp60 response to decrease until eventually concentrations at or below the control response level were reached. So we expected an optimum response curve for hsp60 in *P. acuminatus* upon metal exposure. The optimum response curve was estimated by using a

polynomial regression analysis. This analysis was performed in Microsoft Excel (Windows software) using the built-in solver function.

3.3 Results

3.3.1 Identification of hsp60 in P. acuminatus

The elucidated full-length mRNA sequence of the putative *P. acuminatus* hsp60 and its deduced amino acid translation are shown in Figure 1.

```
   1 CGTCAGGGCCTCACTTCTTAGGAACCTATCGCAACAGAACGTTGGATCGTTGAT   54
  55 TCTAGCTTTGCTCTGTAGCTTCAATAAGCCTCAGAACAAAAAATGCTTCGAACA  108
                                                M  L  R  T
 109 ACTCCAGCCTTGTTCCGTTCGCTGTCTCGACAGTTGCCGCTACTTTCTTCTGCT  162
     T  P  A  L  F  R  S  L  S  R  Q  L  P  L  L  S  S  A
 163 CAATCTCACCGTGGCTACGCCAAGGACCTCAGATTTGGAGCCGAGGGCAGAAAA  216
     Q  S  H  R  G  Y  A  K  D  L  R  F  G  A  E  G  R  K
 217 TCGATGCTCGTCGGTGTTGATTTGCTGGCAGATGCTGTCGCTGTTACAATGGGA  270
     S  M  L  V  G  V  D  L  L  A  D  A  V  A  V  T  M  G
 271 CCCAAGGGTCGCAACGTGATCATTGAACAGTCATGGGGAAGCCCGAAAATCACT  324
     P  K  G  R  N  V  I  I  E  Q  S  W  G  S  P  K  I  T
 325 AAAGATGGTGTCACTGTCGCTAAAGCGGTCGATCTCAAGGACAAGTTTCAAAAT  378
     K  D  G  V  T  V  A  K  A  V  D  L  K  D  K  F  Q  N
 379 CTCGGAGCCAAGCTAGTGCAGGATGTAGCCAACAAGACGAATGAAGTAGCCGGA  432
     L  G  A  K  L  V  Q  D  V  A  N  K  T  N  E  V  A  G
 433 GACGGAACGACGTGCGCCACAGTGCTCGCCCGAGCGATCGCCAAGGAGGGTTTC  486
     D  G  T  T  C  A  T  V  L  A  R  A  I  A  K  E  G  F
 487 GATAACATCAGCAAGGGCGCCAACCCGGTCGAAGTACGGCGAGGGGTGATGCTC  540
     D  N  I  S  K  G  A  N  P  V  E  V  R  R  G  V  M  L
 541 GCCGTCGACACTGTGGTCGCCGAGCTGAAGAAGATGTCCCGGCCCGTGACAACG  594
     A  V  D  T  V  V  A  E  L  K  K  M  S  R  P  V  T  T
 595 CCGGAAGCCATTGCCCAGGTGGCGACCATCTCGGCCAACGGAGATACGGTCATC  648
     P  E  A  I  A  Q  V  A  T  I  S  A  N  G  D  T  V  I
 649 GGCAATCTCATCTCCGAAGCGATGAAGAAGGTGGGTAACAGAGGCGTGATCACG  702
     G  N  L  I  S  E  A  M  K  K  V  G  N  R  G  V  I  T
 703 GTCAAGGATGGAAAGACGCTGACAGATGAGCTTGAAACTGTGGAAGGCATGAAG  756
     V  K  D  G  K  T  L  T  D  E  L  E  T  V  E  G  M  K
 757 TTCGATCGTGGCTATATTTCGCCCTACTTCATCAACTCGAGCAAGGGCGCCAAG  810
     F  D  R  G  Y  I  S  P  Y  F  I  N  S  S  K  G  A  K
 811 GTCGAGTACGAAAAGGCACTCTTCCTTCTCAGCGAGAAGAAAATCAGTCAAGTC  864
     V  E  Y  E  K  A  L  F  L  L  S  E  K  K  I  S  Q  V
 865 CAGGATGTTGTGCCCGCTTTGGAATTGGCCAACAAGTACCGCAAACCGCTCGTC  918
     Q  D  V  V  P  A  L  E  L  A  N  K  Y  R  K  P  L  V
 919 ATTATTGCCGAGGACGTGGACGGCGAAGCTCTGACGACGCTCGTGCTCAACAGG  972
     I  I  A  E  D  V  D  G  E  A  L  T  T  L  V  L  N  R
 973 CTCAAGGTCGGTCTACAAGTGGCGGCAGTAAAGGCGCCAGGCTTCGGCGACAAC 1026
     L  K  V  G  L  Q  V  A  A  V  K  A  P  G  F  G  D  N

1027 CGCAAGAACACACTGCGCGACATTGCTGCGATGACTGGCGCAGCGGTGTTCGGC 1080
     R  K  N  T  L  R  D  I  A  A  M  T  G  A  A  V  F  G
1081 GACGAGGCGAACATGATCAAGCTCGAAGATGTGCAGATCCAGGACTTGGGCGAA 1134
     D  E  A  N  M  I  K  L  E  D  V  Q  I  Q  D  L  G  E
1135 GCGGAAGAGATCACCATCACCAAGGATGACACGCTCATCTTGCGCGGCAAGGGT 1188
     A  E  E  I  T  I  T  K  D  D  T  L  I  L  R  G  K  G
1189 AACTCCGCAGACGTGGAAAAGCGCATGGAGCAGATCCTCGACGAAGTTGAACAT 1242
     N  S  A  D  V  E  K  R  M  E  Q  I  L  D  E  V  E  H
1243 TCCACTTCGGACTACGAGAAGGAGAAGCTGAACGAGCGCCTCGCCAAGCTCAGC 1296
     S  T  S  D  Y  E  K  E  K  L  N  E  R  L  A  K  L  S
1297 AAGGGCGTCGCCGTCCTCAAGGTTGGCGGTGGCAGCGAAGTGGAGGTGAACGAG 1350
     K  G  V  A  V  L  K  V  G  G  G  S  E  V  E  V  N  E
1351 AAGAAGGACCGTGTAACGGACGCGTTGAACGCGACTCGCTGCGCCGTCGAAGAA 1404
     K  K  D  R  V  T  D  A  L  N  A  T  R  C  A  V  E  E
1405 GGCATCGTTCCCGGCGGCGGCGTCGCCTTCCTGCGCACAATTGCCTCCCTGAAA 1458
     G  I  V  P  G  G  G  V  A  F  L  R  T  I  A  S  L  K
1459 GCGCTCAAACCGGCCAACGACGATCAGCTGAAAGGTATTAAAATCGTTGAGAAG 1512
     A  L  K  P  A  N  D  D  Q  L  K  G  I  K  I  V  E  K
1513 GCGTTGCGCATGCCAATCACAACGATCGTGTCGAACGCGGGTGTCGAACCGTAC 1566
     A  L  R  M  P  I  T  T  I  V  S  N  A  G  V  E  P  Y
1567 GCGGTGGTGGAGCAGGTGCTGCAGAACAAGGAGATCAATTATGGCTATGACGCG 1620
     A  V  V  E  Q  V  L  Q  N  K  E  I  N  Y  G  Y  D  A
1621 ATGAACGGCGAGTTCGTCAACATGATCGAAAAGGGAATCGTCGATCCCACCAAG 1674
     M  N  G  E  F  V  N  M  I  E  K  G  I  V  D  P  T  K
1675 GTGATTCGAACGGCGCTGCAAGATGCCGGCTGGTGTGGCTTCATTGTTGGCGACG 1728
     V  I  R  T  A  L  Q  D  A  A  G  V  A  S  L  L  A  T
1729 ACCGAGTGCGTGATCACCGAACAGCCGAAGAAAGACACAGCCACAGCCGGAATG 1782
     T  E  C  V  I  T  E  Q  P  K  K  D  T  A  T  A  G  M
1783 GGCGGCATGGGTGGTGGAATGGGTGGCATGGGCGGTGGAATGGGTGGCATGTTC 1836
     G  G  M  G  G  G  M  G  G  M  G  G  G  M  G  G  M  F
1837 TAATCGTTCCAGAGACATGATGCTATTACCATTTGTTGACTTTGTTGTTATGCA 1890
     *
1891 TTAGGTGACGATTGCGCCATCCTTCCAACGTATTCGTTACTTCACCGTCATTCT 1944
1945 ATCTAGTATCGCGTTAGTTCCGTCTAGTTTGTTGAGTGTAACTTGTTACAATTT 1998
1999 TACATTCTCCAGTGCCCGCGAGAGTAAATTACAGAATATCAAAAAAAAAAAAAA 2052
2053 AAAAAAAAAAAAAAA 3'
```

Figure 1: Full-length hsp60 mRNA of the nematode *Plectus acuminatus*. Depicted is the base sequence with the predicted amino acid translation of the encoded protein. The sequence was submitted to the EMBL/GenBank Libraries under accession number AJ130877. The amino acids encoding the putative mitochondrial targeting signal are underlined.

Both the mRNA and the deduced amino acid sequence showed considerable sequence identity with hsp60s of other organisms, both invertebrates and vertebrates, prokaryots and plants as detected by using BLAST (Altschul *et al.*, 1990; Gish and States, 1993). At the mRNA level, the highest homology was obtained with the fruit fly *Drosophila melanogaster* hsp60 (81%) and hsp60 of the nematode *Caenorhabditis elegans* (80%). At the protein level,

hsp60 of the nematodes *Onchocerca volvulus* (80%) and *C. elegans* (76%) showed the highest homology to the *P. acuminatus* sequence. Relationships between a selection of hsp60 sequences of several organisms based on their homologies at the amino acid level as determined by the computer programme DNASIS (Hitachi Software Engineering Company, Yokohama, Japan) are depicted in Figure 2.

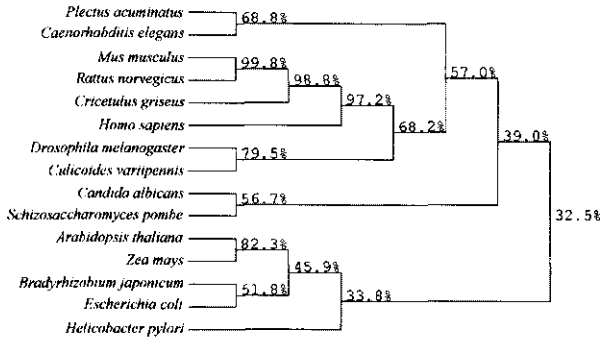

Figure 2: Dendrogram of identities between hsp60 amino acid sequences of several organisms as determined by the computer programme DNASIS (Hitachi Software Engineering Company, Yokohama, Japan). The hsp60 sequences were extracted from BLAST (Altschul *et al.*, 1990) by using the following accession numbers: CAA10230 (*Plectus acuminatus* (nematode)), P50140 (*Caenorhabditis elegans* strain CB1392 (nematode)), CAA38762 (*Mus musculus* (mouse)), P19227 (*Rattus norvegicus* (rat)), P18687 (*Cricetulus griseus* (Chinese hamster)), P10809 (*Homo sapiens* (human)), CAA67720 (*Drosophila melanogaster* (fruit fly)), AAB94640 (*Culicoides variipennis* (biting midge)), AAC34885 (*Candida albicans* (fungus)), CAA91499 (*Schizosaccharomyces pombe* (fission yeast)), P29197 (*Arabidopsis thaliana* (plant)), S20875 (*Zea mays* (maize)), P35861 (*Bradyrhizobium japonicum* (bacterium)), P06139 (*Escherichia coli* (bacterium)) and S61397 (*Heliobacter pylori* (bacterium)).

Using the deduced amino acid translation of the elucidated *P. acuminatus* hsp60, the protein consists of 580 amino acids and its predicted weight is 61.794 kDa with an isoelectric point of 6.16. It contains a mitochondrial targeting signal of 28 amino acids as determined by MitoProt II 1.0a4 (Claros and Vincens, 1996).

The elucidated full-length mRNA sequence of the putative *P. acuminatus* actin shared the highest homology with *D. melanogaster* actin (88%) and the deduced amino acid sequence with actin of the branchopod *Artemia sp.* and the nematodes *Wuchereria bancrofti* and *C. elegans* (98%).

3.3.2 The hsp60 protein response in P. acuminatus upon metal exposure

At the protein level, a significant optimum curve was found for the hsp60 response in *P. acuminatus* after exposure to zinc chloride (Fig. 3) ($p = 0.000767$). At the investigated zinc chloride concentration range, according to the best fitted curve the hsp60 response in *P. acuminatus* increased with increasing zinc chloride concentrations to a maximum of over 8 fold the control response at a concentration of 291 µM zinc chloride, after which the response decreased with further increasing zinc chloride concentrations. The hsp60 concentrations were expected to reach the control level after exposure of the nematodes to 581 µM zinc chloride.

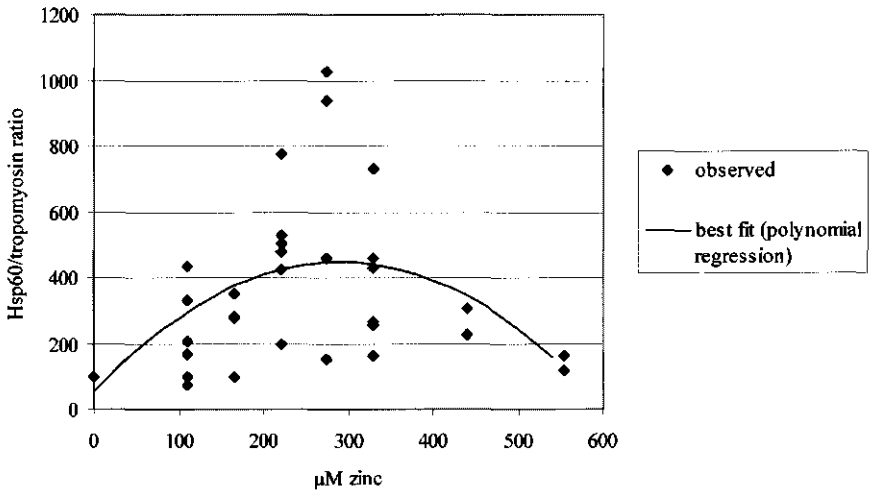

Figure 3: Hsp60 response at the protein level (hsp60 per tropomyosin ratio) in the nematode *P. acuminatus* after 24 hours of exposure to increasing zinc chloride concentrations. Each group of measurements (one concentration range) is calculated as a percentage of the respective control (control =100%). The observed values are represented by black diamonds, the best data fit as obtained by polynomial regression analysis is represented by the curve.

The hsp60 response in *P. acuminatus* also increased with increasing copper chloride concentrations ($p = 0.014$), but the maximum hsp60 induction was not reached within the investigated copper chloride concentration range (results not shown). At the highest investigated concentration of nearly 60 µM copper chloride, the hsp60 response was over 16 fold higher than the control response, though the variation was also very high.

When the nematodes were exposed to cadmium chloride, the average hsp60 response showed an optimum trend with a maximum induction at 27.3 µM cadmium chloride, but this trend was not significant (results not shown).

3.3.3 The hsp60 mRNA response in P. acuminatus upon metal exposure

At the hsp60 mRNA level, in *P. acuminatus* no considerable increase was obtained when compared to control levels and to the protein levels. For comparison, after metal exposure nematode samples were split using one half to detect the hsp60 response at the protein level and the other half to detect the hsp60 response at the mRNA level. After exposure to zinc chloride, at the mRNA level only a slight elevation was observed at the lowest investigated zinc chloride concentration (Fig. 4). At the two highest investigated zinc chloride concentrations, the hsp60 mRNA response even dropped below the hsp60 mRNA control level.

After exposure of the nematodes to copper chloride, an elevation of the hsp60 mRNA response was observed at the lowest investigated copper chloride concentrations (results not shown). This elevation was more pronounced compared to the one of the zinc chloride response, though it was still less than a factor 2 compared to the control hsp60 mRNA response.

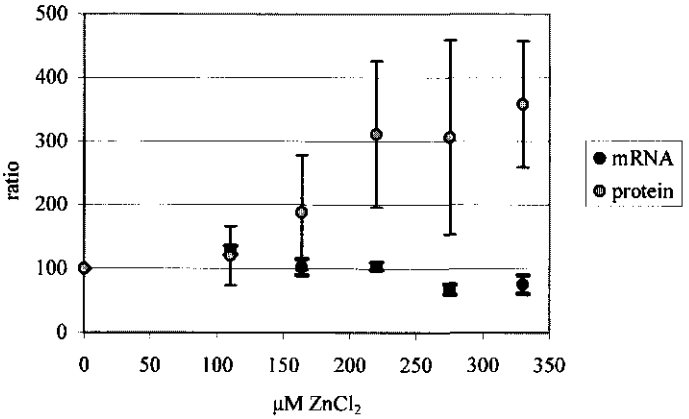

Figure 4: Hsp60 response in the nematode *P. acuminatus* after 24 hours of exposure to increasing zinc chloride concentrations. The samples were split using one half to measure the hsp60 response at the protein level (hsp60 per tropomyosin ratio; grey dots) and the other half to measure the hsp60 response at the mRNA level (hsp60 per actin ratio; black dots). Each group of separate measurements is calculated as a percentage of the respective control (control = 100%). Error bars indicate the standard error of the mean.

3.4 Discussion

3.4.1 Identification of hsp60 in P. acuminatus

In this study, hsp60 in the nematode *P. acuminatus* was identified by its full-length mRNA sequence and deduced amino acid translation. Hsp60 is known to be highly conserved (Schlesinger *et al.*, 1982). This is evidenced by the high homology found between the elucidated *P. acuminatus* hsp60 and hsp60 of other organisms, though this protein is clearly not as highly conserved as some other proteins, e.g. actin. We found that the homology between the *P. acuminatus* actin and actin of other organisms was much higher than the hsp60 homology, especially when taking the deduced amino acid sequence in account.

Hsp60 in *P. acuminatus* is, as expected, a mitochondrial protein as confirmed by the presence of a putative mitochondrial targeting signal. The presence of a putative targeting signal typical for proteins that are translocated to the mitochondrial matrix has also been reported in hsp60 of e.g. *Drosophila melanogaster* (Kozlova *et al.*, 1997), *Trypanosoma cruzi* (Giambiagi *et al.*, 1993), *Culicoides variipennis* (Abdallah *et al.*, 2000) and *Paracentrotus lividus* (Gianguzza *et al.*, 2000). Most probably, this signal will be cleaved resulting in the mature hsp60 protein to be smaller and of less weight than predicted by using the complete deduced amino acid translation of the mRNA.

3.4.2 The hsp60 protein response in P. acuminatus upon metal exposure

After exposure of *P. acuminatus* to increasing zinc chloride concentrations, an optimum curve for the hsp60 response at the protein level was found. This is in agreement with findings by Eckwert *et al.* (1997), Zanger and Köhler (1996) and Guven *et al.* (1994)

concerning the hsp70 response in the isopod *Oniscus asellus*, the diplopod *Julus scandinavius* and a transgenic strain of the nematode *Caenorhabditis elegans* respectively. Eckwert *et al.* (1997) discovered a typical optimum curve in the hsp70 response of *O. asellus* to increasing metal concentrations. This curve could be divided into three zones: In the first zone, the hsp expression was only marginally increased due to exposure to comparatively low metal concentrations; in the second zone, strong hsp induction occurred and the ability of the heat shock system to react to increased metal concentrations reached its climax; and in the third, non-compensation zone, a further increase in metal concentrations resulted in a decline of hsp levels, which could be interpreted as a result of pathological tissue damage (Eckwert *et al.*, 1997). In the present study, the response curve of hsp60 in *P. acuminatus* at the protein level after zinc chloride exposure most probably reflects the second and third zone as described by Eckwert *et al.* (1997). Maybe the first zone is also present, but this cannot be extracted from the present data set. Low zinc chloride concentrations would have to be tested to gain more insight. After exposure of *P. acuminatus* to increasing copper chloride concentrations, the hsp60 response in this animal would possibly follow the same trend as obtained after zinc chloride exposure, but probably only the second zone of the hsp response was (partly) covered by the applied copper chloride concentration range. Therefore, the hsp60 response in this nematode species should also be investigated at higher copper chloride concentrations (> 60 μM) to find the maximum induction (the end of zone 2) and zone 3 of the hsp60 response.

In former research (chapter 2), an increase of the hsp60 protein level in *P. acuminatus* was found to be related to increasing concentrations of cadmium and copper chloride (Kammenga *et al.*, 1998). In the present study, this relationship was confirmed for increasing copper chloride concentrations. However, after exposure to increasing cadmium chloride concentrations, the trend detected in the hsp60 response in *P. acuminatus* in the present research was not significant. This might be due to the fact that in the former research the samples were qualitatively examined, were incubated 2 hours in water instead of 24 hours on agar and at much lower cadmium chloride concentrations compared to the present research. Maybe the cadmium chloride concentrations in the present research were too high to induce a significantly higher level of hsp60 in the nematode and the response had already been quenched (the end of zone 3). The lowest cadmium chloride concentration used was about 10% of the LC_{50} of this toxicant for *P. acuminatus* in water after 72 hours of exposure (Kammenga *et al.*, 1994) and higher than the EC_{20} for the daily reproduction and juvenile period of *P. acuminatus* on 0.5% agar (Kammenga *et al.*, 1996b). In order to reduce the stress caused by the toxicant, the best solution would be to use lower cadmium chloride concentrations and not to decrease the exposure time, because the relatively long exposure time of 24 hours in the present research was chosen in order to minimize the effect of handling stress.

The high variability encountered in the hsp60 response in *P. acuminatus* at the protein level and often found in literature regarding hsp responses is being discussed in the next chapter (paragraph 4.4.2). This high variability implies that many replicates are needed to detect a significant hsp60 response trend at the protein level.

The hsp60 response in *P. acuminatus* at the protein level is possibly a suitable biomarker for metal pollution, but it clearly depends upon the metal(s) and the concentrations involved. Furthermore, the number of repetitions needed to acquire a reliable result is not promoting this biomarker as a quick ready-to-use tool without taking in account its complex kinetics of induction and de-regulation. The effect of confounding factors such as temperature and pH on the hsp60 response of the nematode should also be investigated for the interpretation of this biomarker in various situations.

3.4.3 The hsp60 mRNA response in P. acuminatus upon metal exposure

We found that the hsp60 mRNA level in *P. acuminatus* was giving a less pronounced elevated response compared to the hsp60 protein level in this nematode. If any elevation of the hsp60 mRNA level was detected, it was found at lower metal concentrations compared to the response at the protein level. More extensive testing would be needed to verify these findings. Furthermore, the hsp60 response should be examined at more concentrations in the low concentration range (e.g. < 150 µM zinc chloride), because the hsp60 mRNA peak might be found within this range. The hsp60 mRNA peak might also be found at the concentration range we applied in this study, but after shorter exposure times. Shorter exposure times, however, implicate more influence of handling stress.

Because each hsp family comprises multiple closely related isoforms (Lindquist, 1986) and several hsp60 isoforms have been detected in *P. acuminatus* (Kammenga *et al.*, 1998), it is important to realize that it is not certain that the hsp60 in *P. acuminatus* measured at the mRNA level is the same hsp60 measured at the protein level. If more extensive studies are undertaken to compare the hsp60 response in *P. acuminatus* at the mRNA and protein level, efforts should be made to elucidate this issue.

With regard to the suitability of the hsp60 mRNA response against the hsp60 protein response in *P. acuminatus* as a biomarker for metal stress, the hsp60 mRNA levels in *P. acuminatus* show much less variation compared to the hsp60 protein levels and are therefore much more reproducible. However, possibly the elevation of the response at the mRNA level is not high enough and/or occurs at very low metal concentrations, which would make it inappropriate as a biomarker for metal pollution. The latter has been discussed by Köhler *et al.* (1998) concerning the hsp70 response in the grey garden slug *Deroceras reticulatum* exposed to cadmium- or zinc-enriched food. They investigated the hsp70 response at the mRNA and at the protein level and found that the relative hsp70 mRNA level reached higher than the protein level compared to the control. Furthermore, the mRNA level reacted at much lower metal concentrations compared to the protein level. However, they pointed out that under field conditions the high sensitivity of the hsp70 mRNA level might make it less suitable as a biomarker (Köhler *et al.*, 1998).

In other papers, results concerning the hsp60 mRNA and protein response in a particular organism or tissue of an organism can vary considerably, depending on the organism or organism tissue involved, the stressor and its concentration/level, exposure period and the time between exposure and hsp60 measurements. Nevertheless, usually both the hsp60 mRNA and protein levels are transiently elevated, though the protein response often lags behind the mRNA response and the maximum induction levels are different, which is in agreement with our findings. In human proximal tubule cells, the hsp60 mRNA and protein levels were both elevated after 4 hours of exposure to cadmium chloride to a maximum of 2-3 fold and 4-5 fold the control level respectively 4 hours after removal of the stressor (Somji *et al.*, 2000). The hsp60 levels in this test returned to the control levels 8 hours (mRNA) and 24 hours (protein) after removal of the cadmium chloride. When using non-metal stressors in similar tests, the hsp60 mRNA level always increased quicker, reached the maximum sooner and returned to control values earlier than the hsp60 protein level (Somji *et al.*, 2000). Upregulation of hsp60 mRNA followed by increased hsp60 protein expression has also been found in other investigations with mammals (Neufer *et al.*, 1996; Schett *et al.*, 1997). The rate of hsp60 synthesis generally correlated well with the hsp60 mRNA levels in response to certain stress factors as found in several tests using mammalian cells/tissues (Ovelgönne *et al.*, 1995; Latif *et al.*, 1999; Bajramovic *et al.*, 2000). In the latter study, the enhanced hsp60

protein levels occurred either simultaneously with or shortly (1-2 hours) after hsp60 mRNA upregulation was observed.

Not always the hsp60 mRNA and protein levels follow the same trend: In *Trypanosoma cruzi,* the hsp60 mRNA level increased 6-fold at 37°C compared to 26°C or 42°C, whereas the hsp60 protein level remained essentially constant at these temperatures (Sullivan *et al.*, 1994). In chronically stimulated skeletal muscles of rats the hsp60 protein level increased 3.2 fold, whereas the hsp60 mRNA level did not alter (Ornatsky *et al.*, 1995). One should consider whether the hsp60 mRNA and protein levels are measured at the right time intervals, because the time interval between the induction of the two responses may vary considerably.

In conclusion, in the present study the hsp60 level in *P. acuminatus* increased considerably more at the protein level and occurred at higher metal concentrations when compared to the mRNA level at the investigated metal concentrations. Although the variability at the protein level was much higher compared to the mRNA level, the hsp60 protein response in *P. acuminatus* may have more potential as a biomarker for metal stress than the hsp60 mRNA response.

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CHAPTER 4

Application of the hsp60 biomarker in the nematode *Plectus acuminatus* exposed in the field to metals in a gradient near Avonmouth, UK. *

ABSTRACT - Heat shock proteins (hsps) are potential biomarkers for monitoring environmental pollution. In the former chapters of the present thesis, the hsp60 protein response in the nematode *Plectus acuminatus* was brought to light as a potential biomarker for metal stress in laboratory experiments. In the present chapter, the application of this biomarker was evaluated for the use in field bioassays. Therefore, *P. acuminatus* specimens were transplanted into six field sites along a metal gradient near Avonmouth, UK, and the hsp60 protein response was measured. The response appeared to be significantly higher in the nematodes transplanted into the field site with the lowest metal concentrations compared to the other field sites. The responses of the nematodes in the other field sites did not significantly differ from each other. It can be concluded that the hsp60 response in *P. acuminatus* alone was not a suitable biomarker for heavily contaminated soils. However, this biomarker had indicative value when related to other biomarker responses measured simultaneously in the same field sites (e.g. the hsp70 response in the isopod species *Oniscus asellus* and *Porcellio scaber*). Furthermore, it might be a suitable biomarker for less heavily contaminated soils. This would have to be investigated in field experiments, because laboratory experiments provide no alternative.

* Based on: Arts, M.S.J., Schill, R.O., Knigge, T., Eckwert, H., Kammenga, J.E. and Köhler, H.-R. Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. *Ecotoxicology* (accepted).

4.1 Introduction

The biomarker concept involves the use of biochemical, cellular and physiological parameters as diagnostic screening tools in environmental monitoring (Sanders, 1990). Biomarkers are of special interest to ecotoxicologists, because they have the potential to bridge the gap between chemical analyses for the presence of toxicants and impairments of organismal physiology (Hightower, 1993) before effects can be measured at the organismal level (e.g. changes in life cycle traits) that can lead towards effects at the population and community level in ecosystems. Furthermore, biomarkers indicate the combined effect of toxicants (Dunlap and Matsumara, 1997), which is especially important in field studies where organisms are usually exposed to toxicant mixtures.

A monitoring strategy using biomarkers would be most effective if it incorporated biomarkers to measure the general stress responses for initial screening (tier I), combined with biomarkers for measuring specific responses to help identify the stressors (tier II). For tier I biomarkers, the molecular mechanisms involved in protecting and defending cells from environmental insults have great potential (Sanders, 1990). One of those mechanisms is the cellular stress response. The heat shock response is part of this cellular stress response and is extensively described in chapter 1 and in the introduction of chapter 2 and 3 of this thesis.

Terrestrial invertebrates are important in soil ecological systems. They are inhabiting all soil habitats and play an important role in food webs, in the decomposition of organic material and in soil structuring. Nematodes are the most dominant group of multicellular organisms on earth and are abundant in all habitats and feeding types. For our field experiments, the terrestrial free-living bacterivorous nematode *Plectus acuminatus* (Nematoda, Plectidae) Bastian 1865 was selected because it is ubiquitous in the moderate regions of the world, it can be cultured in the laboratory and proved to be suitable for toxicity tests (Kammenga *et al.*, 1996). Previous work revealed hsp60 to be more suitable to monitor proteotoxic metal effects in *P. acuminatus* than hsp70 (Kammenga *et al.*, 1998). Furthermore, an optimum curve and an increase of the hsp60 protein response in *P. acuminatus* had been detected after exposure to zinc chloride and to copper chloride respectively in laboratory experiments (chapter 3). Therefore, in the field experiments, we focused exclusively on the hsp60 protein response in *P. acuminatus*. The terrestrial free-living bacterivorous nematode *Caenorhabditis elegans* (Nematoda, Rhabditidae) Maupas 1899 was selected because it occurs in the UK, can easily be cultured in the laboratory and is the best characterized nematode species that exists. Because hsp70 appears to be the most potential hsp biomarker in *C. elegans* for metal exposure (e.g. Liao and Freedman, 1998), hsp analysis was only projected onto hsp70 in this nematode species in the field experiments.

The hsp responses in the nematodes were evaluated alongside other biomarkers in other terrestrial invertebrates (e.g. hsp70 in isopods, metallothioneins in snails, earthworms and springtails) which were all incorporated in the same research project (reviewed by Kammenga *et al.*, 2000). The main objective of this project was to validate the biomarkers for assessing the exposure and effects of toxicants (in this case metals) on soil invertebrates in the field. The chosen field locations, where all biomarkers were tested simultaneously, were situated in the Avonmouth area (near Avonmouth), located near the Severn estuary in the UK (see Fig.1). This area is highly contaminated principally from a primary cadmium, lead and zinc smelter, with zinc, cadmium, copper and lead being the most important pollutants. Seven field sites were selected along the metal gradient. The nematodes were deployed in six of them, designated site 1, 2, 2a, 3, 4 and 5 (for their location on a map: see Fig.2). Site 1 was located on the verge of a public footpad, site 2 and 4 on the grass verges of minor roads, site 3 in a pasture, site 2a in an area of oak woodland and site 5 in a sparsely planted oak stand with dense grass cover. Sites 2,

4 and 5 were located on the flat, low lying alluvial area, while sites 1, 2a and 3 were located on the rising ground that adjoins the alluvial plain. The geology and geography of the sites and the characterization of their soils including physicochemical analysis have extensively been described by Filzek *et al.* (in press). Because there was no unpolluted site and therefore no real control site, when possible, of all terrestrial invertebrates included in the research project both resident and transplanted specimens (coming from laboratory cultures or from pristine sites) were investigated. In this way interpretation of the biomarker was enhanced and a possible adaptation of the resident populations to the polluted field sites, resulting in tolerant populations, could be detected. No resident nematodes were sampled because of the impossibility to determine nematodes to the species level without killing or at least heavily stressing them. Therefore, nematodes were cultured in the laboratory and transplanted into the field sites for the evaluation of the hsp biomarker in these animals in field bioassays.

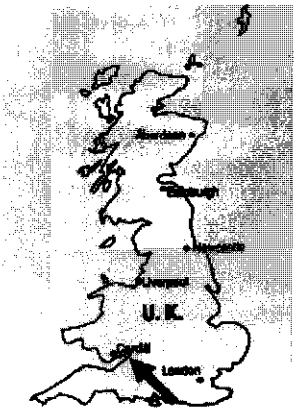

Figure 1: Location of the Avonmouth area (field experiment) in the UK, indicated by the black arrow.

Figure 2: The location of the six field sites in the Avonmouth area where nematodes were transplanted into. The asterisk indicates the location of the smelter.

4.2 Materials and methods

4.2.1 Culturing

The nematode *P. acuminatus* was originally extracted from the top mineral layer of arable soil at the Binnenhaven in Wageningen, The Netherlands, in 1993. Stock cultures have been kept in the laboratory since and a population was reared from one individual of this stock for the use in all the experiments. Rearing took place on proteose pepton agar (0.05% proteose pepton (Difco Laboratories) in 1% or 3% technical agar (Oxoid Limited)) in 6 cm plastic petri dishes (Greiner) sealed with parafilm at 20°C in a climate chamber (Elbanton). Prior to adding the nematodes to the plates, 30 µl of *Acinetobacter johnsonii* strain 210A (the Netherlands Culture Collection of Microorganisms, access number LMAU A130 (Bonting *et al.*, 1992))($2 \cdot 10^8$ cells/ml yeast extract) were pipetted on the plates in a laminar flow chamber (Holten LaminAir), spread with a glass rod and allowed to grow overnight at 28°C to serve as a food source for *P. acuminatus*. *A. johnsonii* was cultured in sterile yeast extract (Difco

Laboratories, 4 g/l) in aerated bottles overnight at 28°C until a concentration of approximately $2 \cdot 10^8$ cells/ml was reached and kept at 4°C until use. The nematode *C. elegans* Bristol strain N2 was reared on nematode growth medium (NGM) agar (Brenner, 1974; Wood, 1988) in 9 cm plastic petri dishes (Greiner) sealed with parafilm at 20°C in a climate chamber (Elbanton). Instead of *A. johnsonii*, *Escherichia coli* strain OP50 was used as a foodsource (Brenner, 1974), from which 75 µl were spread on each agar plate and grown at 37°C overnight.

For transplantation experiments, nematodes and bacteria had to be concentrated. The cultured nematodes were collected by putting the agar with the animals on a wet filter (Hygia milac sw (Hartmann), 220 mm diameter) followed by the extraction procedure for active nematodes (van Bezooijen, 1997). The nematodes were flushed over a 10 µm sieve (40 mm diameter, Laboratory of Nematology, Wageningen) to retain the nematodes whereas the bacteria can pass. The nematodes on the sieve were flushed with autoclaved standard pore water (SPW), which is an aqueous solution of a defined mixture of minerals resembling the interstitial water of sandy forest soils (Schouten and Van der Brugge, 1989). They were flushed into 50 ml plastic tubes (Greiner), centrifuged at 760 g for 2 minutes (MSE super minor centrifuge), the overlaying water removed, the nematodes collected again in 10 ml plastic tubes (Greiner), centrifuged at 760 g for 2 minutes, the overlaying water removed, etcetera until the desired volume was reached. The number of nematodes was counted 3 times in at least 4 subvolumes of 10 µl with a stereomicroscope (Leica wild M3Z, magnification 160). The final density of the nematode suspension for transplantation was approximately 10 nematodes per µl. Bacteria were concentrated by centrifugation at 12,096 g at 16°C for 2 minutes (Beckman Avanti Centrifuge J-25 I). The pellets were washed with SPW and collected in one tube, concentrated again by centrifugation at 12,096 g for 2 minutes, washed again etcetera until a thick suspension (approximately 10^{10} bacteria per ml) was obtained.

4.2.2 Transplantation

To transplant the nematodes, a minicontainer system described by Eisenbeis (1993) was modified. In this system, nematodes were exposed in small plastic containers (16x11 mm) in a minicontainer holder. The minicontainers were filled with soil, which was collected with a soil core sampler at the 6 sites near Avonmouth where nematodes were to be transplanted into. The upper 5-15 cm of these soil cores, collected in September 1997, were stored at 4°C. In May 1998 any stones, roots and visible animals were removed from the cores and from each site 200 gr of soil were weighted in a glass petridish, all at room temperature. The petridishes with soil were heated in a microwave oven (Samsung 800W) for 1 minute at full power to kill any resident organisms. Then the soil was dried at 80°C overnight and the dry weight was determined. The original wet weight was regained by adding the suspension of concentrated bacteria (see paragraph 4.2.1) mixed with yeast extract to the dried soil ($2 \cdot 10^8$ bacteria/ gr dried soil). Final mixing took place overnight on a roller bench (Luckham multimix mm1 (Denly Instruments Ltd), speed 1.5) at room temperature. One side of the minicontainer was closed by glueing a disc (25 mm diameter) of nylon transfer membrane (Nytran 0,45 µm (Schleicher & Schuell)) with 2-component glue (Kombi snel Bison) on the container rim and drying it overnight at room temperature. Subsequently, three-quarter of the container was filled with the prepared soil. 100 µl of nematodes (approximately 1000 nematodes in total) were pipetted on top of this soil and covered with the soil until the container was filled. Then the container was closed by glueing a nylon transfer membrane disc on the rim of the open end and drying it for 2 hours, all at room temperature. The filled

minicontainers (for an example: see Fig. 3a) were put in the holes of the minicontainer holder (see Fig. 3b), 12 containers per holder, 1 holder per site. Of the 12 minicontainers, 11 contained soil of the corresponding site with bacteria and nematodes and one soil of the corresponding site with only bacteria, the latter serving as a negative control. Each minicontainer was considered to be a statistical unit.

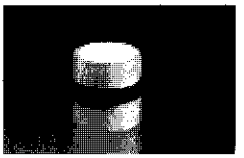

Figure 3a: A filled minicontainer

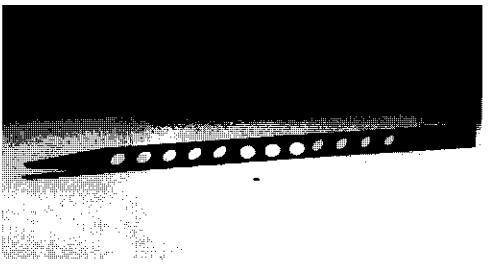

Figure 3b: A minicontainer holder with 3 minicontainers

The filled minicontainer holders were loosely wrapped into plastic foil to prevent evaporation and kept at 20°C until transportation. At the field location, the minicontainer holders were buried horizontally into the soil with the center of the membranes of the minicontainers all at a depth of 10 cm. Soil samples were taken just near the buried minicontainers to measure the pH and the 0.01 M $CaCl_2$ extractable metal content of the soils at the exact location of nematode exposure (nematode microhabitat). After 5 days, the minicontainer holders were retrieved, loosely wrapped into plastic foil and transported to Wageningen, The Netherlands. The content of each minicontainer, treated as a separate unit, was flushed with SPW into a beaker (250 ml), put on a wet filter (Hygia milac sw (Hartmann), 220 mm diameter) and treated as described in paragraph 4.2.1 until the final weight of each sample was 20 mg. The samples were frozen in liquid nitrogen and kept at -80°C until further use.

The field experiments were conducted in May 1998 (pilot study; *P. acuminatus* and *C. elegans*) and in May 1999 (*P. acuminatus*). Except for the 5 days the nematodes were exposed in the field sites, handling and transport also took about 5 days.

4.2.3 Laboratory experiments

The *P. acuminatus* transplantation units, used in the field experiment, were also exposed in laboratory experiments to enhance the interpretation of the field results. In one experiment the hsp60 response of *P. acuminatus* was measured. Because the retrieval of the nematodes from the soil of the different sites appeared to differ, in another experiment the recovery of *P. acuminatus* was measured.

For each test 5 minicontainers were filled with soil of the respective sites, bacteria and nematodes as described in the former paragraph and exposed in a climate chamber (Elbanton) at 15°C for 10 days. The minicontainer holders were loosely wrapped into plastic foil to prevent evaporation during exposure. After exposure, the nematodes were retrieved and concentrated as described before. The nematodes for the hsp60 measurements were frozen in liquid nitrogen and kept at -80°C until further processing (see paragraph 4.2.4). The nematodes for measuring the recovery were fixed in 37% formaldehyde (Merck) with 3% acid fuchsin (Fluka Chemie) for at least one day. The fixed nematodes were flushed over a 10

μm sieve (9 cm diameter, Laboratory of Nematology, Wageningen) and counted by using a stereomicroscope (Leica Wild M3Z, magnification 160).

4.2.4 Immunological analysis

The hsp60 response in *P. acuminatus* was measured by using one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) and subsequent Western blotting as described in chapter 3 (paragraph 3.2.5). The monoclonal antibodies mouse anti-human hsp60 clone LK-2 (cat. no. SPA-807, StressGen Biotechnologies Corp., Victoria, Canada) and mouse anti-*Globodera rostochiensis* tropomyosin (De Boer *et al.*, 1996) were applied as primary antibodies (both 1:1000 diluted). Tropomyosin, a protein of the striated muscle filaments, served as an internal standard. Alkaline phosphatase-conjugated rat anti-mouse antibodies (Jackson ImmunoResearch laboratories, West Grove, Pennsylvania, USA) were employed as secondary antibodies (1:5000 diluted). Grey values of the blot bands were measured by densitometric image analysis (Magiscan image analysis system (APPLIED IMAGING, Tyne & Wear, UK) Programme Genias). The data were processed using the computerprogramme MIDAS (APPLIED IMAGING, Tyne & Wear, UK) and the hsp60 per tropomyosin value (h/t value) was calculated for each sample. Although analyses comprise hsp60 of bacteria associated with the nematodes by adhesion to the cuticula or by ingestion by the nematodes, the respective stress protein level can be assigned to the nematodes themselves due to the comparatively small mass of the bacteria.

Because no *C. elegans* had been recovered after transplantation into the field, no hsp70 measurements were performed.

4.2.5 Statistics

Differences in h/t values of the nematodes between the different sites were statistically evaluated using a *t*-test or ANOVA. For the laboratory tests with Avonmouth soil, the correlation between the h/t values and the recovery was calculated using the canonical analysis of MANOVA.

4.3 Results

4.3.1 Field experiments

Of the transplanted nematodes, about 25% of the original number of *P. acuminatus* was retrieved from the containers which were buried in site 1, 2, 2a, 3 and 4. It appeared that 3% or less was recovered from the containers which were buried in site 5, suggesting that the conditions in site 5 were too toxic for *P. acuminatus*. From the containers with *C. elegans* no nematodes were retrieved at all, not from any of the six sites in which they were buried.

After processing the *P. acuminatus* samples as described, the negative controls did not exhibit any hsp60 response. The *P. acuminatus* samples from site 5 neither showed any response, probably due to the small number of the nematodes retrieved from this site, resulting in amounts of target proteins below the detection level. The pooling of samples from site 5 did not improve the detectability.

The hsp60 response measured in *P. acuminatus* transplanted into site 1, 2, 2a, 3 and 4 is displayed in Figure 4. The h/t values of the nematodes exposed in site 1, 2a, 3 and 4 did not

significantly differ from each other. In contrast, the h/t values of the nematodes exposed in site 2 were significantly higher compared to the ones of the other sites ($p < 0.035$). The variability of the h/t values was also the highest in this site. The metal concentrations measured at the exact location of nematode exposure (nematode microhabitat) are shown in Table 1. When comparing these metal concentrations to the hsp60 response, the high h/t values of *P. acuminatus* exposed in site 2 coincided with the lowest metal concentrations measured. The metal concentrations measured at the nematode microhabitats clearly differed from the ones measured randomly at the sites (Filzek *et al.*, in press) and did not correspond to the ranking of the sites related to the distance of the smelter.

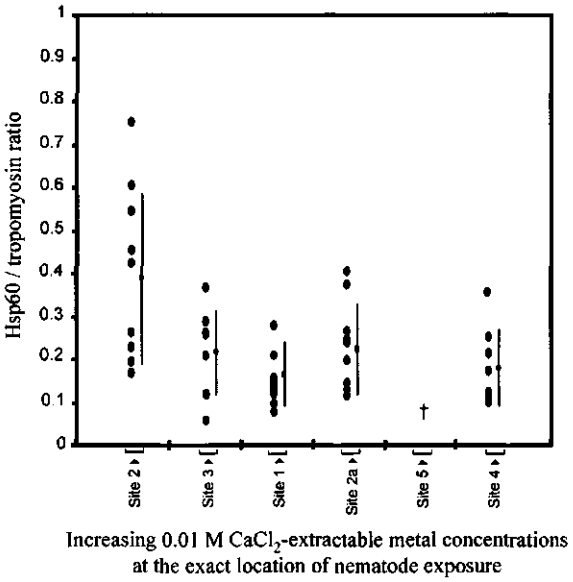

Figure 4: Hsp60/tropomyosin values in the nematode *P. acuminatus* transplanted from the laboratory into the field sites near Avonmouth, UK. Small dots with whiskers represent means ± SD, big dots individual data. Individual data consist of the contents of one minicontainer (> 25 nematodes). Sites are arranged according to increasing metal concentrations measured at the exact location of nematode exposure.

Table 1: The 0.01 M $CaCl_2$ extractable metal concentrations (µM/kg dw soil) at the exact location of nematode exposure (nematode microhabitat). Pb concentrations were not included in the table, because they were negligibly low.

	site 1	site 2	site 2a	site 3	site 4	site 5
Zn	196.7	2.9	458.5	114.8	2094.9	1889.4
Cu	0.4	0.3	0.6	0.7	4.1	4.4
Cd	1.9	0.2	8.5	2.5	10.9	30.4
total	199.1	3.3	467.5	118.1	2109.9	1924.2

4.3.2 Laboratory experiments

In the laboratory experiments exposing *P. acuminatus* to soil of the sites near Avonmouth, the h/t values did not exhibit any significant differences (Fig. 5). In this experiment only one h/t value was obtained from the nematodes exposed to the soil of site 4. Apparently, retrieval of the nematodes from the soil of this site was too low to detect any target protein in 4 of the 5 minicontainers (in contrast to the field experiment, where the number of nematodes retrieved after transplantation into site 4 was high enough to measure the hsp60 response). Despite the recovery of the nematodes transplanted into site 5 in the field experiment being too low to measure any target protein, in the laboratory experiment there was enough recovery of the nematodes exposed to the soil of this site to determine the hsp60 response.

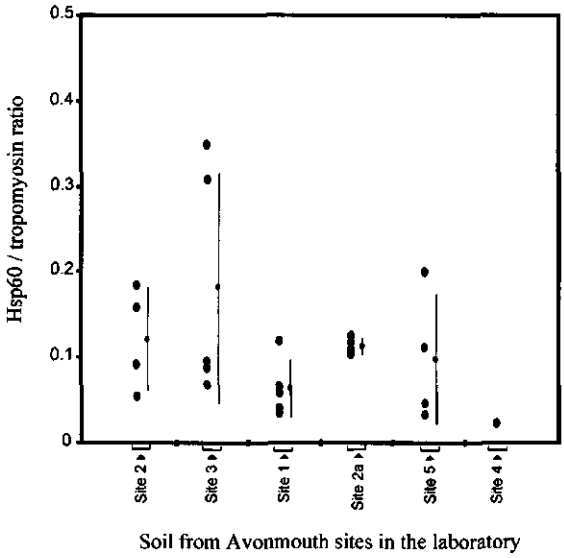

Figure 5: Hsp60/tropomyosin values in the nematode *P. acuminatus* after 10 days of exposure at 15°C in the laboratory in soil of 6 field sites near Avonmouth, UK. Small dots with whiskers represent means ± SD, big dots individual data. Individual data consist of the contents of one minicontainer (> 25 nematodes). Sites are arranged according to increasing metal concentrations measured at the exact location of nematode exposure.

The recovery experiment in the laboratory to investigate the apparent differences in retrieval of *P. acuminatus* from the soil of the different sites revealed that the recovery was significantly lower from the soil of site 4 compared to the other sites ($p< 0.04$) and from site 2a compared to site 1, 2 and 3 ($p<0.045$) (Fig. 6). There was no significant difference in the recovery of *P. acuminatus* from the soil of site 1, 2 and 3. The retrieval of the nematodes from the soil of site 5 was much higher than expected (not significantly different from the soil of site 1, 2, 3 and 2a). When ranking the recovery of *P. acuminatus* to the zinc concentrations measured at the nematode microhabitats, a clear trend emerges: the higher the zinc concentration, the lower the recovery (Fig. 7). When ranking the recovery to the cadmium concentrations the same trend was observed, though not so pronounced as with zinc (results not shown).

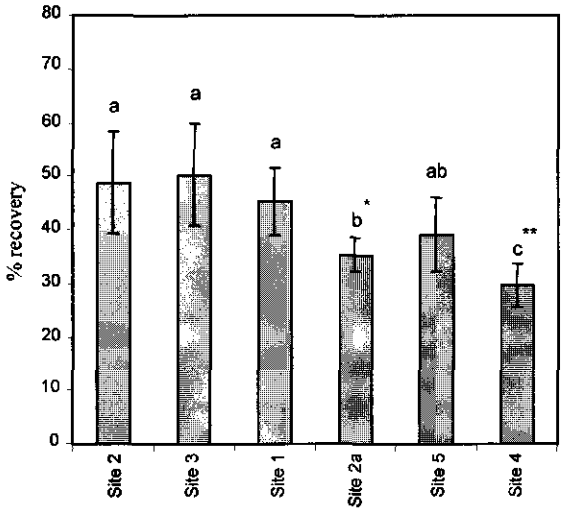

Figure 6: Percentage recovery of the nematode *P. acuminatus* after 10 days of exposure at 15°C in the laboratory in soil of 6 field sites near Avonmouth, UK. Sites are arranged according to increasing metal concentrations measured at the exact location of nematode exposure. Significant differences are indicated by different letters (*t*-test, $p<0.045$).

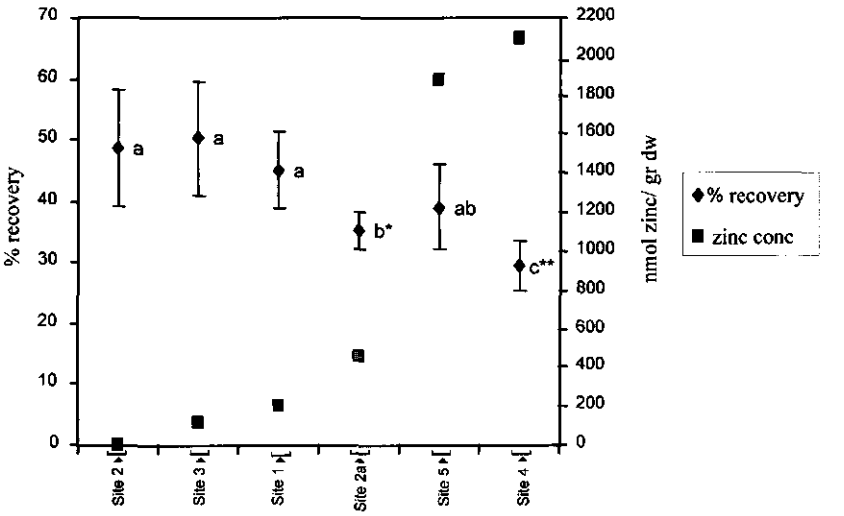

Figure 7: Percentage of recovery (diamonds with whiskers representing means ± SD) of the nematode *P. acuminatus* after 10 days of exposure at 15°C in the laboratory in soil of 6 field sites near Avonmouth, UK, versus the 0.01M $CaCl_2$ extractable zinc concentrations (squares) measured at the exact location of nematode exposure. Different letters indicate significant differences (*t*-test, $p<0.045$). Sites are arranged according to increasing zinc concentrations in the respective microhabitat of the nematodes.

With the canonical analysis of MANOVA a positive correlation was found between the recovery and the hsp60 response of *P. acuminatus* exposed under equal conditions in the laboratory to soil of the sites near Avonmouth (standardized canonical coefficient for recovery = 1.2077, for hsp60 = 0.4410, $p < 0.0230$ (Pillai's Trace)). This meant that a higher recovery correlated to a higher hsp60 response.

4.4 Discussion

4.4.1 Field and laboratory experiments

In the field experiment, the nematode *P. acuminatus* displayed the highest hsp60 response in the microhabitat with the lowest metal concentrations (measured at the exact location of nematode exposure): site 2. The zinc concentration was at least about a factor 40 higher in the nematode microhabitats of all other sites (see Table 1). It seemed that the metal concentrations in all the sites, except for site 2, provoked quenching of the hsp60 response in *P. acuminatus*, which might explain the low h/t values. Also the isopod *Oniscus asellus*, transplanted into the Avonmouth sites, showed rather low hsp70 levels in response to the microcosms buried at the most contaminated sites 4 and 5 in relation to other less polluted sites. Both findings are in agreement with the results of Wheelock *et al.* (1999), who investigated the hsp60 response in the rotifer *Brachionus plicatilis* upon exposure to increasing concentrations of a water accomodated fraction (WAF) of crude oil. They found that only the extremely low levels of WAF resulted in values of hsp60 significantly higher than controls. They suggested that the ability to inhibit hsp60 induction may indicate a more toxic response then producing elevated levels of this protein. Even earlier, it had been reported that organisms exposed to high concentrations of pollutants often showed a decreased hsp70 content compared to lower concentration treatments in laboratory experiments (Guven *et al.*, 1994; Eckwert *et al.*, 1997). Eckwert *et al.* (1997) found that in response to increasing metal concentrations, the hsp70 level in the isopod *O. asellus* first increased to a certain 'maximum of induction' and subsequently declined, which they thought was most probably due to a pathological impact on the animals' tissues. From our field experiment with nematodes it appeared that the recovery of *P. acuminatus* in site 5 was lower than in the other sites and from the laboratory experiments that the recovery of the nematodes from the soil of site 4 and 2a was lower compared to the other sites. When recovery had been influenced, one may assume that the physiology of the organisms was already affected to such extent that their ability to actively pass the filter was hampered and therefore the hsp60 response had certainly been quenched. This assumption was supported by the positive correlation found between the recovery and the h/t values in the laboratory experiment by using MANOVA. The investigation of the hsp60 response is only interesting when recovery is not significantly lower compared to control circumstances. When recovery is slighty higher compared to the control, the hsp response could render interesting information. A slighty higher recovery can be connected to sublethal stress, because the nematodes try to escape the adverse conditions. In recent studies, many nematode taxa exhibited stimulation at low copper concentrations and inhibition at higher copper concentrations regarding recovery efficiency (T. Bongers, pers. comm.).

The h/t ratios obtained in the field experiment (see Fig. 4) were generally much higher compared to the h/t ratios obtained in the laboratory (see Fig. 5) and the patterns also differed. Furthermore, the conditions in the soil of site 5 were far less toxic and of site 4 were more

toxic for *P. acuminatus* in the laboratory experiment compared to the field experiment. The differences between the results of the field and laboratory experiments must be caused by the nematode transport, burial and/or climatological circumstances. Apparently, these factors are of paramount importance for determining the ultimate toxic effects on the nematodes. It also shows that the results of the laboratory experiments cannot be extrapolated to the field. Therefore, laboratory tests could not replace the field experiments.

C. elegans was not retrieved after transplantation into the field, though this species had been recovered after exposure in the transplantation units in the laboratory. Apparently, the circumstances of the field experiment (transport, burial and climatological influence) had a negative effect on the mobility (and possibly vitality) of this nematode species. Because *C. elegans* had been maintained in the laboratory for decades, it might have lost its ability to cope with field conditions.

4.4.2 Variability in hsp values

In both our field and laboratory experiments, the variability of the hsp60 response was very high. Therefore, the differences found between sites were mostly not significant. We often encounter this phenomen in literature regarding hsp responses. For example, Pyza *et al.* (1997) found a high variability in the hsp70 response of the centipede *Lithobius mutabilis* to increasing pollutant concentrations in field and laboratory experiments, resulting in differences being mostly not significant. Wheelock *et al.* (1999) also reported that any statistically significant trend was masked by the large standard errors in the hsp60 response of *B. plicatilis* after exposure to increasing WAF concentrations. Köhler *et al.* (1999) found that hsp70 expression in the springtail *Tomocerus flavescens* could vary by more than 100% among specimens from the same site or exposed to the same laboratory conditions. They suggested that this large variability among replicates might be due to individual differences in transcription rates of *hsp* genes and patchiness in distribution of metals and individuals and food. In the present study these potential causes might be applied to explain the variability in the hsp60 response in *P. acuminatus*, though the patchiness in distribution of metal, individuals and food must be regarded for the nematodes on a microscale. According to Köhler *et al.* (1999), patchiness in food distribution could lead to temporary food shortage, potentially resulting in temporary starvation of soil invertebrates. In our study, food shortage and its distribution might have played an important role in the response of the nematodes. In laboratory tests it had been observed that the growth of *A. johnsonii* on agar was hampered by high metal concentrations. We think that the conditions in several field sites might have been too toxic for *A. johnsonii*, which could have resulted in food shortage and consequent additional stress for *P. acuminatus*. This indirect toxic effect could have affected the overall results, but maybe there was also variation between the minicontainers within the sites or even within a minicontainer.

Because most hsp responses are the result of the combination of various stress factors, it is important to gain more insight into the effect of other stress factors, including temperature and pH, on the hsp60 response in *P. acuminatus*. Moreover, it would also be important to investigate the transiency of this response in order to improve the interpretation of the data and to determine the optimal exposure time. Furthermore, it is possible that in our laboratory experiment concerning the hsp60 response in *P. acuminatus* any significancy in the differences would have emerged by enlarging the sample size, allthough this remains very questionable.

4.4.3 Evaluation of hsp60 in P. acuminatus as biomarker in field experiments

In fact, the question remains whether hsps are good biomarkers for pollutants in terrestrial field sites. Up to now terrestrial field research on hsps, including the present one, did not render substantial evidence to support the assumption that hsps can serve as a ready-to-use tool which can be applied without consideration of their rather complex kinetics of induction and de-regulation. Only with the earthworm *Lumbricus rubellus* in metal contaminated soils, upregulation of hsp70 and hsp90 in resident populations and of hsp60, hsp70 and hsp90 in transplanted populations was registered to be more or less dose dependent, though these studies were qualitative and not quantitative and transplantation experiments were performed in the laboratory with soil and animals from the field sites (Mariño *et al.*, 1999). The other exception is the hsp70 induction in *O. asellus* populations in the field, but this study already considered a more-than-additive action of a metal mixture (Köhler and Eckwert, 1997). However, these findings should not result in the conclusion that hsps per se are no feasible biomarkers but rather encourage scientists to consider non-monotonous concentration-response relationships curves with maxima and minima and multifactorial action of confounding factors in the field. In studies like the present one, the analytical investigation of the interaction of biochemical processes following an optimum type of response can detect response patterns beyond statistical significance which are undoubtedly necessary to understand the aspects of ecological constraints of a biochemical stress response and to allow an application and correct interpretation of biochemical marker studies in retrospective risk assessment in the field.

4.5 Conclusions

The hsp60 response in the nematode *P. acuminatus* alone is not a suitable biomarker for heavily contaminated soils such as all the sites in Avonmouth except for site 2. However, the quenched hsp60 response in the nematode is of indicative value, only when related to a less sensitive biomarker in a proper suite of biomarkers. Whether the hsp60 response in *P. acuminatus* is a suitable biomarker for less heavily contaminated soils remains to be investigated. This will have to be investigated in field experiments, because laboratory experiments provide no alternative. The nematode *C. elegans* is not a suitable test species for transplantation into the field.

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CHAPTER 5

Multi-generation cadmium effects at the population level: Fitness maximisation and optimal allocation in the nematode *Caenorhabditis elegans**

ABSTRACT – A life-history model of the nematode *C. elegans* was developed to calculate fitness maximisation in populations exposed to cadmium chloride during multiple generations. It was shown that maximum fitness depended strongly on the trade-off between sperm maturation time and juvenile development. Once *C. elegans* was exposed to cadmium chloride, fitness decreased during the first generation. After exposure of consecutive generations, fitness increased slightly but significantly compared to the first exposed generation, while various life-cycle traits were strongly affected. The life-history modelling of *C. elegans* showed that cadmium decreased fitness by impairment of juvenile development. The sperm maturation time remained constant. After long-term exposure of multiple generations, *C. elegans* counteracted the effect on juveniles by growing faster and increasing reproduction and fitness. This chapter illustrates that the combination of detailed knowledge of the life-cycle and life-history modelling provides insight into the underlying mechanisms of toxicant induced life-cycle changes and fitness consequences.

* Based on: Kammenga, J.E., Arts, M.S.J. and Doroszuk, A. (2000). Multi-generation effects at the population level: fitness maximisation and optimal allocation in a nematode. In: J. Kammenga and R. Laskowski (Eds.) *Demography in Ecotoxicology*. John Wiley & Sons Ltd, Chichester, UK, pp 163-177.

5.1 Introduction

Within ecotoxicology there is a growing interest into multi-generation effects of toxic compounds at the population level. Increasingly it is realised that the outcomes of relatively short-term toxicity studies may not be used for predicting long-term demographic effects. Postma and Davids (1995) showed that although chironomid populations were exposed to NOEC values of cadmium, multi-generation exposure resulted in a population decline. Considerable attention has been paid to the correlation between short-term individual level effects, e.g. on reproduction or growth, and longer-term population dynamic impacts. For instance, Halbach *et al.* (1983) examined population ecology of rotifers exposed to pentachlorophenol and concluded that relatively small effects at the individual level led to large fluctuations at the population level after some time. Meyer *et al.* (1987) studied the relationship between sublethal effects at the individual level and population responses for cladocerans. They found that when the animals were exposed to heavy metals, population parameters were relatively insensitive or at least equally sensitive compared to individual parameters.

The presence of toxicants in the environment is a selective force and therefore a toxicological stress may be responded to by genetic changes at the population level. During long-term exposure periods over multiple generations, organisms may become tolerant to the toxicant and trade-off mechanisms among life-history traits may become apparent as a result of this. Maintenance of such tolerance mechanisms may require extra resources, also called 'cost of tolerance', which in turn may result in a diminished fitness in comparison to non-tolerant genotypes in unpolluted environments.

In addition to genetic adaptation, phenotypic plasticity plays an important role in tolerance mechanisms involving rapid reversible changes (Hoffman and Parsons, 1991). An example of genetic adaptation is provided by the research on the midge, *Chironomus riparius*. Analysis of the effects of long-term exposure to cadmium in this organism revealed an adaptive change in life-history characteristics due to selection to toxicants and alterations in population growth dynamics (Postma, 1995). Previous studies also showed the ability of species to adapt to a toxic stress by having plastic life-cycle traits (Kammenga, 1995). Although adaptation to toxicants has already been shown for some invertebrate species, such as the fruit fly *Drosophila melanogaster*, three springtail species, the isopod *Porcellio scaber* (Posthuma and van Straalen, 1993) and *C. riparius* (Postma, 1995), there is still lack of insight into toxicant-induced genetic and phenotypic changes in life-history traits and fitness consequences. To fully explore and understand the impact of a toxicant on continuously exposed populations during multiple generations, life-history modelling is an essential tool.

Effects at the population level are mediated through effects on fitness, which is defined by the root of the Euler-Lotka equation. Fitness maximisation is achieved by an optimal combination of life-history traits, which is strongly influenced by trade-offs among these traits. Trade-off relationships are strongly dependent on the ambient environment and therefore unique combinations of fitness values and trade-off relationships exist under different environmental conditions. The main objective of this chapter is to study the change in fitness under toxic stress in a multi-generation experiment and to calculate maximum fitness values based on optimal trade-off combinations of life-history traits.

The bacterivorous soil nematode *Caenorhabditis elegans* was used as a model organism for a multi-generation cadmium toxicity study because of its relatively short life-cycle and its known trade-off relationships between various traits. Populations consist primarily of hermaphrodites that reproduce by self-fertilisation. Males arise spontaneously due to meiotic

non-disjunction, with a frequency of 0,0002 (<1/500) (Wood, 1988) and mate with hermaphrodites, producing progeny that have males and females in equal numbers.

The life-cycle is as follows (Fig. 1): Eggs develop into four consecutive larval stages (L1 – L4) that are punctuated by moults. During the L4 stage, germ cells undergo meiosis and differentiate into mature sperms. At the fourth moult, sperm production stops and simultaneously oocytes start to be generated. The number of oocytes fertilised depends on the number of sperm produced (Hodgkin and Barnes, 1991). Once fertilised, the oocytes develop and after a few hours the new eggs are laid. Depending on the environmental conditions, the reproductive period lasts for a couple of days and thereafter non-reproducing adults may live on for days or even weeks.

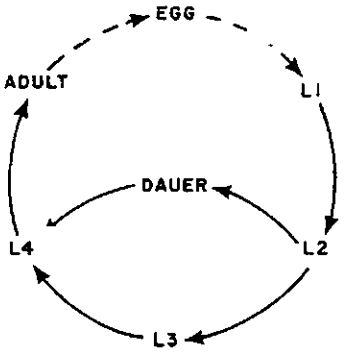


Figure 1. Hermaphroditic life-cycle of the nematode *C. elegans*. L1 – L4 are juvenile stages. The dauer stage is a cryptobiotic stage which occurs when the nematode suffers from food deprivation.

5.1.1 Life-history model

Fitness was calculated from the basic Euler-Lotka equation:

$$\int_0^\infty e^{-rt} \cdot l(t) \cdot b(t)dt = 1 \qquad \text{(eq. 1)}$$

where fitness is defined by r, t is age, l is survivorship, and b is the number of offspring per hour per hermaphrodite. In the Euler-Lotka equation it is assumed that survivorship is time-invariant, but previous studies indicated that survival of *C. elegans* is time-dependent. Survivorship can adequately be described by a Weibull distribution function, where survival $l(t)$ is given by:

$$l(t) = e^{-\ln 2(t/LT_{50})^c} \qquad \text{(eq. 2)}$$

where LT_{50} is the median survival time, t is age and c is a shape parameter of the survival curve.

For further life-history analysis we follow Barker (1992), assuming that after egg hatching individuals first grow during a period g, after which they mature sperm during period s and then switch to production of oocytes at age $g + s$. The period $g + s$ is defined here as the pre-reproductive period. The age at which sperm will run out, d, is equal to the age at switching, $g + s$, plus an age increment, $f(s)$, which is the time required to use up all the sperm that was produced during time s.

According to Barker (1992) and Hodgkin and Barnes (1991), there is a trade-off between sperm maturation time (sperm production) and oocyte production in *C. elegans*. It was found that a delay in egg production was caused by the production of more sperm, which led to higher fertility. Assuming constant sperm production, this trade-off is described by $f(s) = k.s/b$. The constant k is set by the physiology of the animal and was estimated by Hodgkin and Barnes as 12.5. Because s determines this trade-off strongly, we would like to know the maximum fitness value possible at different combinations of s and g where $s + g$ is kept constant. Combining equation (1) and (2) over the period $g + s$ to $g + s + f(s)$ give:

$$\int_{g+s}^{g+s+f(s)} e^{-rt} \cdot e^{-\ln 2(t/LT_{50})^c} \cdot b \; dt = 1 \qquad \text{(eq. 3)}$$

Fitness was calculated with the software package of Mathcad 5.0 using the 'root' function. In order to estimate the correct combination of g and s, we have calculated all combinations and selected the optimal combination, which leads to a maximum fitness.

5.2 Materials and methods

5.2.1 Nematode culturing

C. elegans Bristol strain N2 was used. It was cultured at 20°C in plastic Petri dishes (9 cm diameter, Greiner) on Nematode Growth Medium (NGM) agar with a lawn of *Eschericia coli* strain OP50 as a food source (Brenner, 1974; Wood, 1988).

E. coli was grown in yeast extract at 37°C overnight and kept at 4°C until further use. 90 µl of this bacterial suspension was pipetted on the surface of the agar, spread with a glass rod and left at 37°C overnight to grow.

5.2.2 Multi-generation experiment

At t = 0 two replicate populations were started in separate Petri dishes (9 cm diameter, Greiner). One replicate was used as a control (no cadmium added; hereafter called 'control populations') and in the other replicate dishes, cadmium was added (12 mg $CdCl_2 \cdot l^{-1}$ agar; hereafter called 'cadmium populations'). Cadmium chloride was obtained from Merck, Schuchardt, Germany. For practical reasons, a stock solution of a concentration of 1 g $CdCl_2 \cdot l^{-1}$ millipore water was made in a plastic tube and stored thereafter at 4°C for a maximum of 1 month. The concentration was based on previous toxicity results, indicating that at 12 mg $CdCl_2 \cdot l^{-1}$ agar large sublethal effects on *C. elegans* could be expected without high mortality. After 1 week the populations were randomly sampled for a few thousand individuals and each sample was split in two: One part was stored at -80°C and the second part was transferred to a newly prepared dish. All dishes contained a lawn of *E. coli* and were stored at 20°C in the dark. These manipulations were carried out for 21 weeks.

For life-history analyses, the following frozen samples were selected (two replicates of each sample): (a) control populations after t = 1 week; (b) control populations after t = 21 weeks; (c) cadmium populations after t = 1 week; (d) cadmium populations after t = 21 weeks.

The populations were placed on Petri dishes and left for one week to recover from the freezing stress. The concentrations of $CdCl_2$ in the NGM agar, 0 $mg \cdot l^{-1}$ and 12 $mg \cdot l^{-1}$, corresponded to the ones used before freezing for populations kept formerly on unpolluted and polluted NGM agar, respectively. Subpopulations were then transferred onto new Petri

dishes with the corresponding concentrations of $CdCl_2$ and after 3 or 4 days, depending on the developmental stage of the nematodes, the measurement of life-history traits was started.

5.2.3 Life-history analysis

Individual life-history traits were measured in multi-well plates with lids (Greiner 662160). 70 µl droplets of NGM agar were pipetted on the inner side of the lid opposite the wells, which were filled with 0.5 ml water. To each NGM agar droplet less than 1 µl *E. coli* suspension was added. The plates were incubated at 37°C overnight and kept at 4°C until further use.

The pre-reproductive period (time from egg hatching until full ovary development) was measured on multiplates with the NGM agar containing corresponding concentrations of $CdCl_2$. Estimation of the reproductive period, life span, daily reproduction and total reproduction was obtained by observations of at least 12 individuals from each population (from the fourth larval stage until death) kept separately on Petri dishes (3 cm diameter) with agar containing corresponding concentrations of cadmium and *E. coli.*

For body size assessment, nematodes of a defined developmental stage were used. It was decided to take into consideration only young adults that had 1-4 eggs visible in the body. The data of body length and width were then recalculated to wet weight, using Andrassy's formula (Freckman, 1982):

$$W = (w^2 \cdot L)/(1{,}6 \cdot 10^6) \qquad \text{(eq. 4)}$$

where W = wet weight (µg), w = widest width (µm), L = total length of the nematode (µm).

5.2.4 Statistical analysis

Results were analysed with the statistical package SAS (Anonymous, 1990). The influence of cadmium on different life-history traits was tested using analysis of variance (ANOVA). Replicate populations were tested for differences in variance (based on at least 12 individual recordings per population) and, if they were not significantly different, the replicates were pooled and treated as one group. The average values of this data set were then used to calculate fitness using equation 3. Longevity data were used to estimate survival curves using the algorithm PROC LIFETEST. Subsequent survival values were used in a least-squares method with a non-linear regression procedure (PROC NLIN) to estimate LT_{50} and the shape parameter c in the Weibull distribution curve. Non-parametric log-rank and Wilcoxon tests (for testing differences in large and small survival times, respectively) were applied to analyse differences between the survival curves.

Fitness values were analysed as follows: The optimal values of s and g that gave maximum fitness values were used to calculate a range of fitness values using individual reproduction rates. Subsequent fitness values were bootstrapped using PROC MULTTEST, which allowed analysis of fitness values using Student's t-test.

5.3 Results

For all life-history traits measured, it appeared that there were no significant differences between the two replicates within each treatment. However, to illustrate the variation, the observed values and standard deviations for all traits are shown for each replicate in Figures 2 to 4.

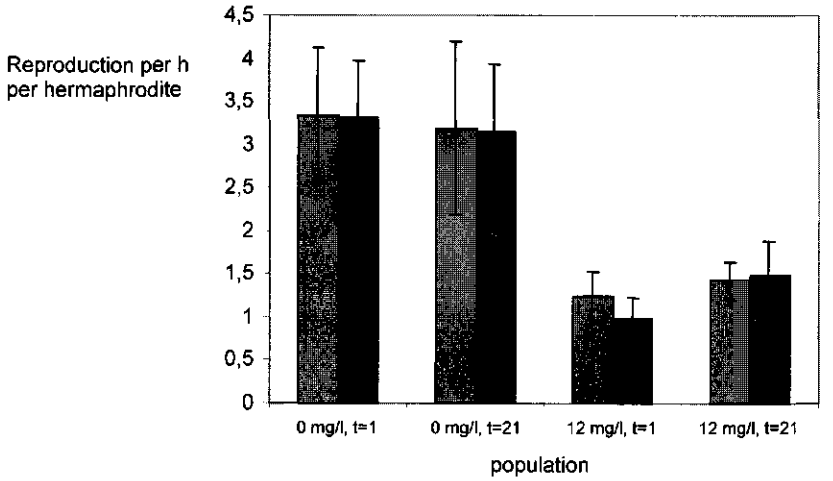


Figure 2: The influence of long-term and short-term exposure to cadmium chloride (12 mg/l agar) on the reproduction rate of *C. elegans* in comparison to control populations (0 mg/l). Time *t* is in weeks. Bars are averages ± SD of each replicate population (light grey: population A, dark grey: population B).

Figure 2 shows that after both short-term (1 week) and long-term (21 weeks) exposure, cadmium appeared to have a severe impact on the reproduction rate. The nematodes taken from the long-term cadmium populations had a higher reproduction rate compared to those taken from the short-term cadmium populations (F=10.5, p=0.003). This was achieved by increasing the total number of progeny rather than shortening the reproductive period, because the reproductive period in both the long- and short-term populations were not significantly different within the cadmium treatment (F=0.3, p=0.59) (Fig. 3). The reproductive period of nematodes from the cadmium-exposed populations was significantly longer in comparison to the control populations for both short- (F=106.3, p=0.0001) and long-term groups (F=50.9, p=0.0001).

Figure 4 shows that the pre-reproductive period measured in the cadmium populations was about 20 hours (≈ 35%) longer than in the control populations (F=409, p=0.0001 for short-term populations; F=439, p=0.0001 for long-term populations, respectively).

Figure 5 shows the survivorship of *C. elegans* for the different treatments. Nematodes in cadmium-exposed populations lived for a shorter time than control populations. The life span in the long-term cadmium populations was significantly shorter than both the long-term (log-rank: $\chi^2 = 15.6, p = 0.0001$; Wilcoxon: $\chi^2 = 16.9, p = 0.0001$) and short-term control populations (log-rank: $\chi^2 = 8.7, p = 0.003$; Wilcoxon: $\chi^2 = 11.9, p = 0.0005$).

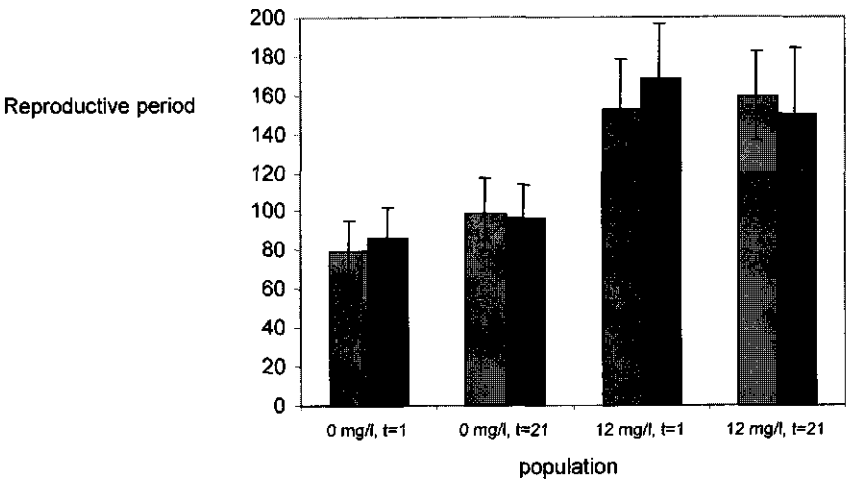


Figure 3: The influence of long-term and short-term exposure to cadmium chloride (12 mg/l agar) on the reproductive period of *C. elegans* in comparison to control populations (0 mg/l). Time *t* is in weeks. Bars are averages ± SD of each replicate population (light grey: population A, dark grey: population B).

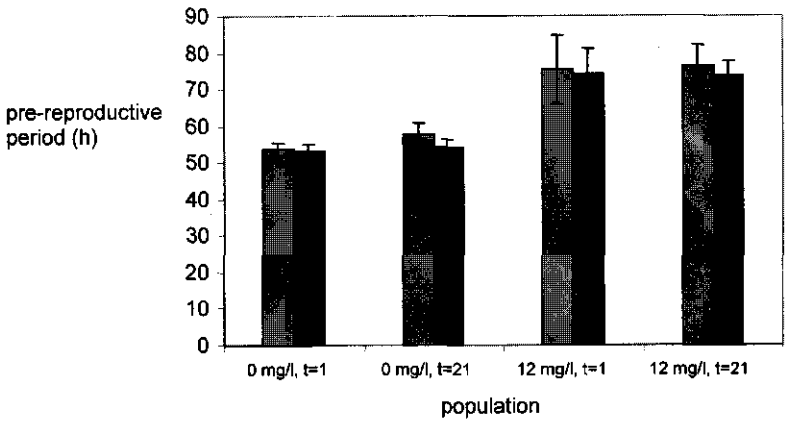


Figure 4: The influence of long-term and short-term exposure to cadmium chloride (12 mg/l agar) on the pre-reproductive period of *C. elegans* in comparison to control populations (0 mg/l). Time *t* is in weeks. Bars are averages ± SD of each replicate population (light grey: population A, dark grey: population B).

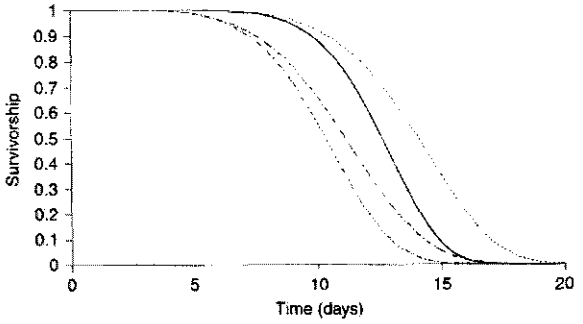


Figure 5: The influence of short-term and long-term exposure to cadmium chloride (12 mg/l agar) on the survivorship of *C. elegans* in comparison to the control populations. ——: control short-term; ·····: control long-term; — — : short-term cadmium; — · — · : long-term cadmium.

The average experimentally obtained values of the life-history traits (except for the reproductive period) were used to calculate fitness using equation 3. Maximum fitness was estimated for different combinations of g and s. For example, in the short-term control populations, $g + s = 53.5$ days (see Table 1). Various combinations of g and s led to 54 days (for numerical reasons Mathcad only accepts discrete values, so 53.5 is rounded to 54), but resulted in different fitness values. Appendix 1 shows the matrix of all different combinations for the short-term control populations. Each element is a fitness value and the diagonal of grey-coloured elements represents the fitness values where $g + s = 54$ days.

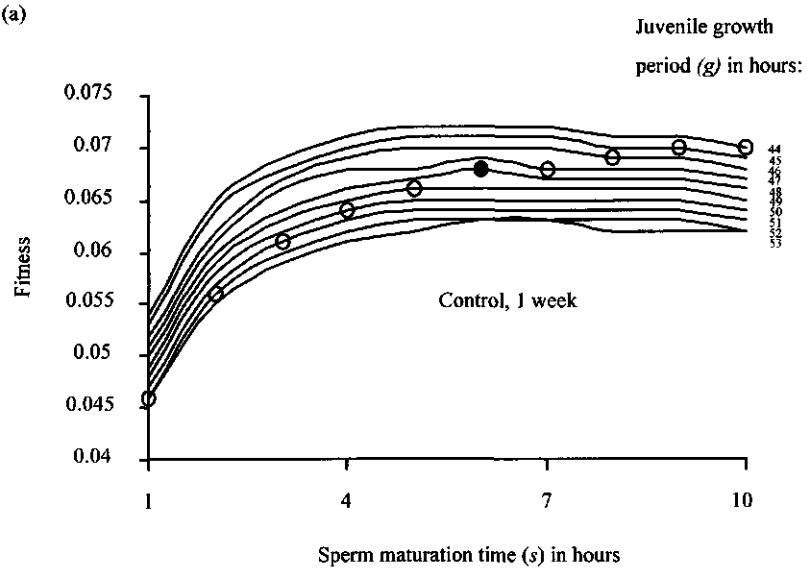
Table 1: Parameters (SD in parentheses) used for calculating fitness r according to equation 3. The allocation constant k was constant (12.5) for all treatments.

	Control (1 week)	Control (21 weeks)	Cadmium (1 week)	Cadmium (21 weeks)
Juvenile growth period in hours ($g + s$)	53.5 (1.7)	55.7 (3.3)	75.8 (8.5)	75.3 (5.7)
Reproduction per hour (b)	3.3 (0.7)	3.1 (0.8)	1.1 (0.3)	1.5 (0.3)
Shape parameter of Weibull survival curve (c)	7.3 (0.9)	6.3 (0.4)	4.8 (0.5)	5.3 (0.4)
Median survival time (LT_{50}) in hours	302.4 (4.8)	336.9 (2.6)	266.4 (3.8)	247.2 (2.6)

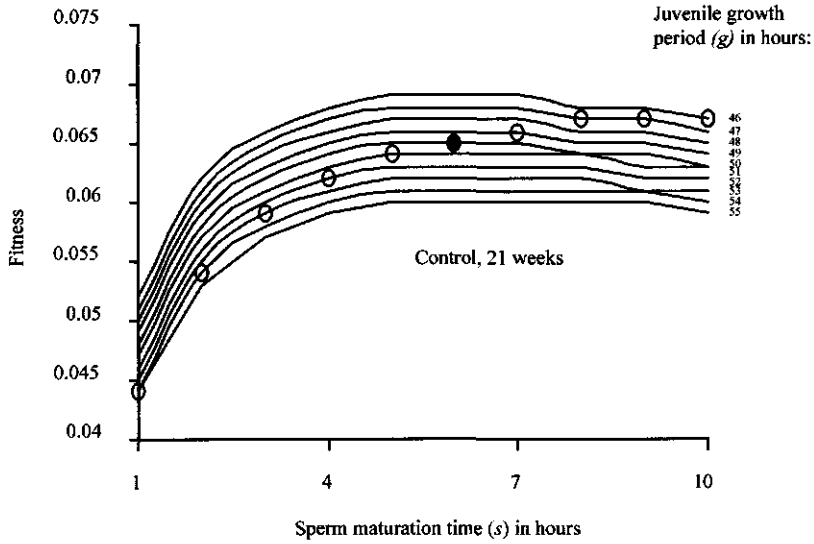
Figure 6 (a-d) gives a graphic representation of the matrix with the sperm maturation time s on the horizontal axis and fitness on the vertical axis. Different curves were drawn for each different value of g. The open dots are the fitness values where $g + s = 54$ days. A black dot indicates where fitness is maximised, hence this combination of g and s returns the highest fitness value (maximum fitness was only designated to the dots which were right on top of a curve). In the short-term control populations, the optimal combination was $s = 6$ and $g = 48$

days, resulting in a bootstrapped fitness value $r = 0.068 \pm 0.002$ (Fig. 6a). In the long-term control populations, $g + s = 56$ days with $s = 6$ and $g = 50$ days being optimal giving a fitness value $r = 0.065 \pm 0.003$ (Fig. 6b).

(a)



(b)



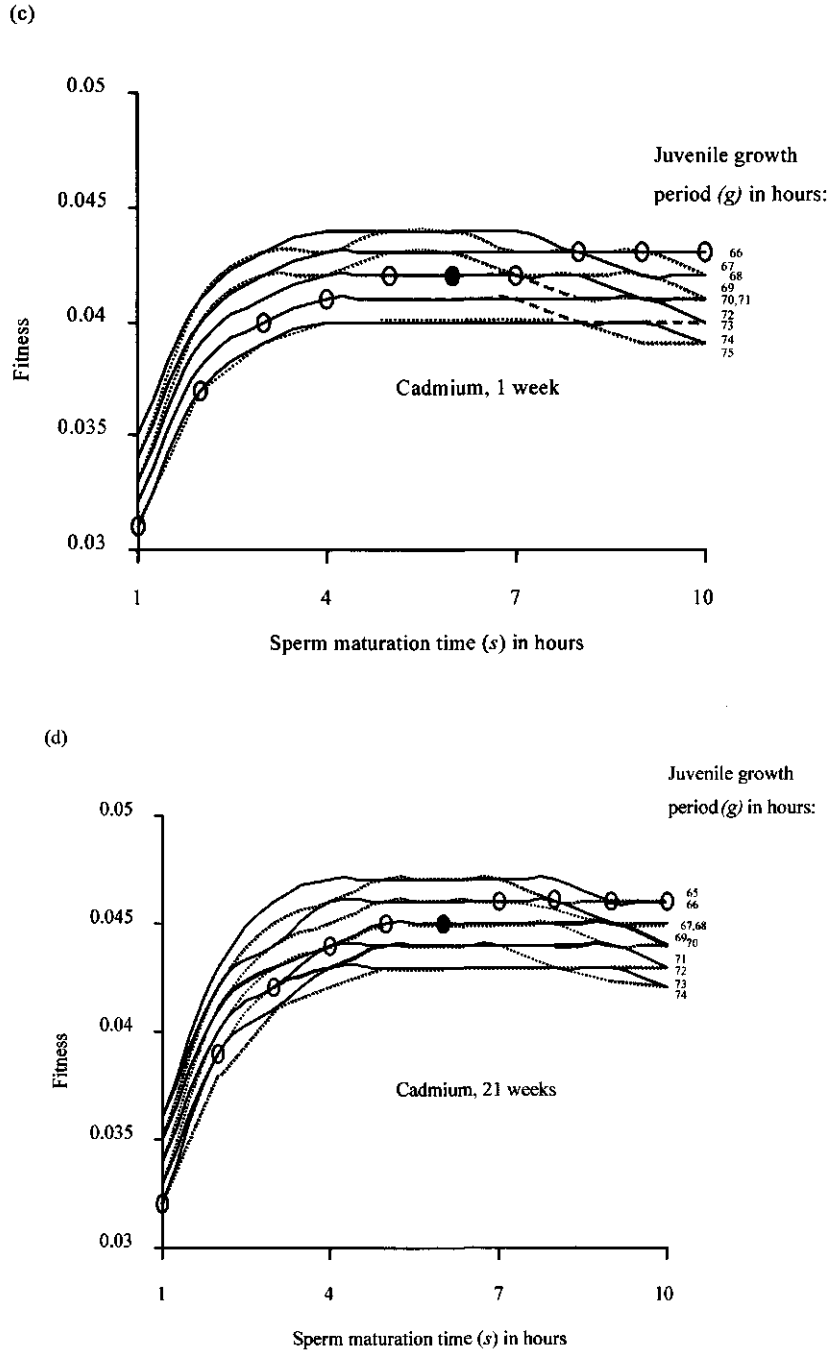


Figure 6: Fitness of *C. elegans* at different combinations of the juvenile growth period (g) and sperm maturation time (s). For more explanation, see text. (a) Short-term control; (b) long-term control; (c) short-term cadmium; (d) long-term cadmium.

Cadmium decreased fitness in both the long- and short-term populations. Fitness maximisation in the short-term populations was obtained when $s = 6$ days and $g = 70$ days (together giving 76 days) and a bootstrapped fitness value $r = 0.042 \pm 0.002$ (Fig. 6c). In the long-term cadmium populations $g + s = 75$ days, with $s = 6$ days and $g = 69$ days giving a fitness $r = 0.045 \pm 0.002$ (Fig. 6d).

After bootstrapping, fitness values of the long- and short-term control and cadmium populations were significantly different from each other ($p = 0.0001$). Importantly, fitness values were also significantly different between long- and short-term populations for cadmium populations ($p = 0.0001$).

The body weight measured in young adults was in general lower in cadmium-exposed populations compared to the control populations (Fig. 7). The difference between short-term control and cadmium populations was significant ($F = 18.17$, $p = 0.0001$). In the long-term cadmium populations an increase could be noticed compared to the short-term cadmium populations ($F = 6.4$, $p = 0.01$).

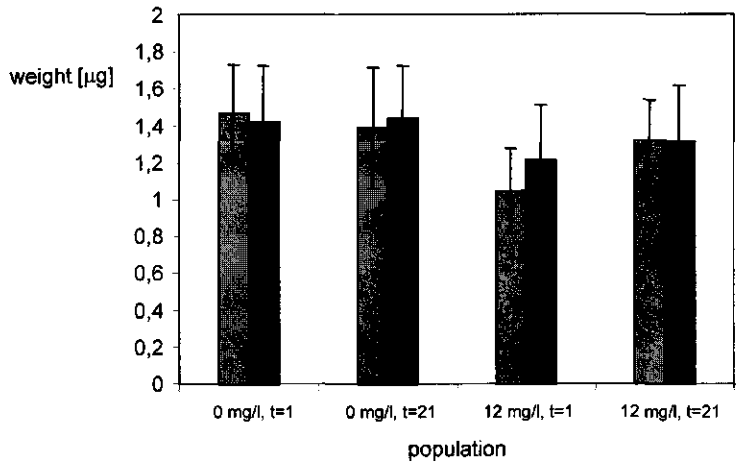


Figure 7: The influence of short-term and long-term exposure to cadmium chloride (12 mg/l agar) on body weight of *C.elegans* in comparison to the control populations (0 mg/l). Bars are averages ± SD of each replicate population (light grey: population A, dark grey: population B).

5.4 Discussion

In the control populations no differences were observed for life-cycle traits and fitness between short- and long-term populations. This agrees with the fact that *C. elegans* does not suffer from any inbreeding depression and populations can be maintained in the laboratory for years without significant life-cycle changes. Compared to the control treatment, short-term exposure of *C. elegans* to cadmium resulted in a prolonged juvenile growth period and a smaller body size at the age of maturity. Furthermore, reproduction rate and survivorship decreased, whereas the reproductive period was longer. The overall impact was a diminished fitness. This result was comparable to the impact of cadmium on the bacterivorous nematode *Plectus acuminatus* in a single generation experiment (Kammenga *et al.*, 1996). The

mechanisms underlying the impact of cadmium can be explained by a paper of Popham and Webster (1976), in which they reported that short-term exposure to cadmium decreased reproduction and reduced body size of *C. elegans* as a result of 'physiological starvation'. The starvation was caused by the influence of cadmium on the uptake and metabolism of nutrients.

We found that continued exposure to cadmium over multiple generations did not change the duration of the juvenile growth period. It was observed, however, that juveniles grew faster. Thus, mature hermaphrodites were larger when egg production started and subsequently reproduction rate increased, resulting in a higher fitness. The larger number of offspring and body size in the long-term cadmium populations suggests that the uptake of nutrients recovered. It indicates the possible presence of mechanisms that arrest the effect of cadmium on nutrient uptake and metabolism, such as the induction of metal-binding proteins or metallothioneins. Liao and Freedman (1998) studied the impact of cadmium on gene transcription in *C. elegans*. Steady-state levels of mRNA expression changed 2-6-fold in response to cadmium exposure. The translated amino acid sequences of several clones were identical to the stress proteins metallothionein-1 and hsp70 and some collagens and rRNAs. It seems therefore reasonable to assume that induction of these proteins counteracts the effect of cadmium, resulting in increased performance of the animal.

The fact that reproduction rate increased with larger body size is in agreement with allometric rules, according to which large individuals within the same species reproduce more than smaller ones (for an example see Hess, 1993). A similar allometric relationship was also found for the closely to *C. elegans* related nematode species *Caenorhabditis briggsae* in a study on food dependency of population growth rate (Schiemer, 1982).

The life-history analyses point out that the sperm maturation time s was 6 days for all short- and long-term treatments (Fig. 6) and that cadmium did not affect sperm production. It can be calculated that $f(s) = 23$ and 24 for the short- and long-term control populations respectively, and $f(s) = 68$ and 50 for the short- and long-term cadmium populations respectively. Considering that $f(s)$ is the time required to use up all the sperm, these findings indicate that cadmium severely affected the fertilisation process of oocytes, resulting in a longer reproductive period. The larger body size and increased reproduction in the long-term cadmium populations are in line with the shorter $f(s)$ of 50 days. It indicates that the time required to use up all the sperm was shorter and subsequently reproduction rate increased.

The values of r are comparable to those found for the closely related species *C. briggsae* (Schiemer, 1982), where r varied between 0.02 and 0.05, depending on food conditions. Also survivorship of the control populations was in agreement with reports by Johnson and Hutchinson (1993) on the life span of *C. elegans*. In the cadmium populations fitness values slightly differed between long- and short-term populations indicating that, although there were significant differences in life-cycle traits, fitness could be maintained at a stable level. It is assumed that the life-cycle changes observed are primarily due to the toxic effect of cadmium. Because multiple generations were exposed, genetic adaptation may have occurred. Life-history theory predicts that reduced adult survival or reproductive success will select for earlier maturation and increased reproductive effort early in life (Charlesworth, 1980). However, this could not be observed in the experiments described here, because nematodes from both control and cadmium populations were not studied under the same conditions. To gain insight into possible genetic adaptation and selection, one should investigate the long-term cadmium populations under non-stressed (i.e. no cadmium present) conditions and perform back crosses with the last generation and the first generation to study possible segregation of life-history traits.

It should be noted that in the present study we assumed that the allocation constant k, which defines the relative costs of egg to sperm, was set at 12.5 for all control and cadmium treatments. This value was taken from Hodgkin and Barnes (1991), who described a *C. elegans* mutant that delays egg production and uses this time to produce more sperm. The ratio of eggs not produced to sperm produced during this period was about 12.5. Barker (1992) studied the relationship between k and survivorship for different life-history scenarios (exponential and linear survivorship and trade-off) and concluded that for a linear trade-off (such as we assumed in this chapter, i.e. $f(s) = k.s/b$) a value of $2 < k < 500$ had very little effect on survivorship. Hence, in the case that cadmium may change the value of k we believe that the outcomes of the study will not be altered given the results by Barker (1992).

So far only very few papers have been published on multi-generation effects of toxicants (Le Blanc, 1982; Postma and Davids, 1995). The present results can be compared with those obtained by Postma and Davids (1995), who conducted an experiment where chironomids were exposed to cadmium for nine consecutive generations. At relatively high cadmium levels they observed a decreased reproduction rate after three to four generations, which was most likely the result of cadmium toxicity. After this period, reproduction rate increased again, which could be explained by acclimation.

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Appendix 1: Matrix of all different combinations of *g* and *s* for the short-term control population of *C. elegans*.

	g									
s	44	45	46	47	48	49	50	51	52	53
1	0.054	0.053	0.052	0.051	0.050	0.049	0.048	0.047	0.046	0.046
2	0.065	0.064	0.062	0.061	0.060	0.059	0.058	0.057	0.056	0.055
3	0.069	0.068	0.067	0.066	0.064	0.063	0.062	0.061	0.060	0.059
4	0.071	0.070	0.069	0.068	0.066	0.065	0.064	0.063	0.062	0.061
5	0.072	0.071	0.070	0.068	0.067	0.066	0.065	0.064	0.063	0.062
6	0.072	0.071	0.070	0.069	0.068	0.066	0.065	0.064	0.063	0.063
7	0.072	0.071	0.070	0.069	0.067	0.066	0.065	0.064	0.063	0.063
8	0.071	0.070	0.069	0.068	0.067	0.066	0.065	0.064	0.063	0.062
9	0.071	0.070	0.069	0.068	0.067	0.066	0.065	0.064	0.063	0.062
10	0.070	0.069	0.068	0.067	0.066	0.065	0.064	0.063	0.062	0.062

CHAPTER 6

Discussion and conclusions

Biomarkers can predict effects of toxicants on cellular function, which might lead to changes in the physiology and/or histology of an organism. These changes possibly exert effects on life-cycle traits, which could lead to changes in fitness, which might eventually lead to extinction of the population. This cascade is represented in Figure 1.

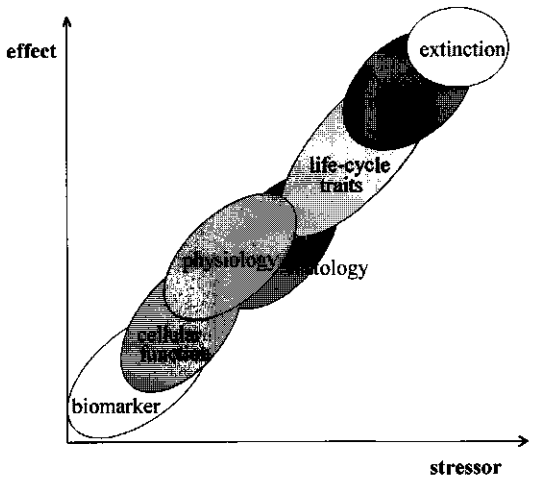

Figure 1: Schematic representation of potential sites of responses of increasing organisational level after exposure to a stressor.

The biomarker evaluated in this thesis, the hsp60 response in the nematode *Plectus acuminatus* (chapters 2-4), is an example of the very beginning of this cascade, the fitness consequences evaluated in *Caenorhabditis elegans* in the multi-generation experiment (chapter 5) is to be found nearly at the end. The stressor was in our case the presence of a metal.

The response of biomarkers to toxic stress only has ecological relevance if the response can be linked to either exposure levels of the toxicant (biomarker of exposure) or effects at higher organisational levels (biomarker of effect) (Kammenga *et al.*, 2000). A biomarker is only of

interest at a very early stage of contamination (low stressor level), because its induction should already become evident far below the concentration level where other sublethal effects become apparent. A biomarker of effect should be correlated with those sublethal parameters or with other toxicological endpoints. Alterations in fitness indicate that harm is inflicted at a very high level of organisation, which is ecologically of the utmost relevance.

6.1 The hsp60 response in P. acuminatus: Biomarker of exposure, but also of effect?

The hsp60 response in *P. acuminatus* to increasing metal concentrations is a biomarker of exposure (see chapter 2-4). To gain some insight into a possible correlation between the hsp60 response in *P. acuminatus* and other toxicological endpoints, some literature data on life-cycle trait alterations in this nematode species upon metal exposure (summarized in Tables 1 and 2) and other literature data are compared to our data. One should bear in mind that the exposure time for the hsp60 and life-cycle trait experiments widely differed (24 hours versus lifetime).

Table 1: Significant effects of copper on life-cycle traits of *P. acuminatus* on 0.5% agar at 20°C (Kammenga and Riksen, 1996).

Life cycle trait	Copper concentration (μM)	Effect
Juvenile mortality	101	100%
Juvenile survival	from 20	↓
Daily reproduction	from 20 47.8	↓ EC20
Length reproductive period	from 30	↓
Survival reproductive period	from 9.7	↑
Length juvenile period	till 40; from 60	↓;↑
Fitness	till 20; from 60	↑;↓

From the qualitative measurements of the hsp60 response in *P. acuminatus* to copper (chapter 2), it appeared that this response was over 1600 times more sensitive than the EC20 for the daily reproduction and 326 times more sensitive than the survival over the reproductive period, which was the most sensitive life-cycle trait to copper measured (see Table 1). When investigating the hsp60 response of the nematode quantitatively (chapter 3), a significant increase of the response was detected with increasing copper concentrations, but it was impossible to discern which copper concentration was the lowest observed effect concentration (LOEC). The best fit as obtained by polynomial regression analysis concerned the whole data set and not the individual data separately. Because the variability between the individual data was high, the use of the data set as a whole was more appropriate to analyse the response. The same conclusion could be drawn for the hsp60 response of *P. acuminatus* to increasing zinc concentrations (chapter 3). Effects of zinc on the occurrence of the genus *Plectus* in soil tests has been described by Korthals *et al.* (1996). The EC50 after 1-2 weeks of exposure was 52 mg zinc per kg soil. In these soil tests, it also appeared that the genus *Plectus* was about twice as sensitive to zinc compared to copper concerning its occurrence. The hsp60 response of *P. acuminatus* was more sensitive to copper than to zinc in laboratory tests on agar with an exposure time of 24 hours (chapter 3). It is difficult to compare these data due to the different types of substrate, different exposure times and different parameters measured, but it showed that a zinc effect had been observed at another level than the

biomarker level. There were no other data available on *P. acuminatus* or on the genus *Plectus* concerning zinc exposure.

For cadmium, the LC50 value (72 hours, in water) for *P. acuminatus* was 107 µM, for *C. elegans* 131 µM (Kammenga *et al.*, 1994). From the qualitative measurements of the hsp60 response in *P. acuminatus* to cadmium (chapter 2), it appeared that this response was 115 times more sensitive than the EC20 for the daily reproduction (see Table 2). However, when investigating the hsp60 response of the nematode quantitatively (chapter 3), no significant trend was observed at the investigated cadmium concentrations. Possibly, the cadmium concentration range was too high to observe any significant increase of the hsp60 response in *P. acuminatus*. Compared to the qualitative experiments, concentrations were higher and the nematodes were exposed for 24 hours on agar instead of for 2 hours in water.

Table 2: Significant effects of cadmium on life-cycle traits of *P. acuminatus* on 0.5% agar at 15°C ([a]) or at 20°C ([b]) (Kammenga *et al.*, 1996).

Life cycle trait	Cadmium concentration (µM)	Effect
daily reproduction	7.6[a] 4.4[b]	↓ (22%) EC20
length reproductive period	7.6[a]	↓ (45%)
length juvenile period	7.6[a] 8.9[b]	↑ (7.5%) EC20
fitness	7.6[a]	↓ (40%)

[a] = 15°C, [b] = 20°C

From the available information as reported here, no conclusions could be drawn concerning the hsp60 response in *P. acuminatus* being a biomarker of effect, apart from being a biomarker of exposure. Only maybe with copper chloride, some correlations were found between an increase in the hsp60 response (both qualitatively and quantitatively) and life-cycle trait alterations in *P. acuminatus*.

My laboratory experiments call for extra consideration regarding metal availability. Metal was adsorped by agar and bacteria, causing the actual concentrations to be lower than the nominal ones. This has been shown for cadmium in former research (Kammenga, 1995), where the original 5 µM cadmium was reduced to 4.5 µM in agar without bacteria and to 3.6 µM in agar with bacteria after 24 hours. Because in these tests 0.5% agar was used, in my experiments (chapter 2 and 3, 1% agar) more metal would have been bound to the agar. Furthermore, the metals were applied as chloride salts, which might influence the total stress load and/or change the toxicity of the metal. For *Folsomia candida*, the zinc toxicity was enhanced by the presence of chloride (Posthuma *et al.*, 1998). The importance of anion type and concentration when using metal salts in toxicity experiments was also demonstrated for the earthworms *Eisenia fetida* and *Eisenia andrei* (Hartenstein *et al.*, 1981; Weltje *et al.*, 1995). On the other hand, *C. elegans* was demonstrated to be very tolerant to salinity: Up to 264 mM NaCl and up to 154 mM KCl did not cause any significant mortality of this nematode in K-medium (Khanna *et al.*, 1997).

6.2 Responses at higher organisational levels: Life-cycle parameters and fitness

Exposure of *C. elegans* to 65.4 µM cadmium on agar at 20°C (chapter 5) resulted in changes in life-cycle traits comparable to the changes observed for *P. acuminatus* (Kammenga *et al.*, 1996; see Table 2), except for the reproductive period being significantly prolonged in *C. elegans* but reduced in *P. acuminatus*. This implies that, besides many similarities, also some differences were observed between the life-cycle trait responses of two nematode species of the same feeding group to the same toxicant. In the *C. elegans* experiment multiple generations were tested, thus allowing the detection of trade-off mechanisms among life-history traits and fitness consequences, leading us to the ultimate effect of the toxicant with the highest ecological relevance as depicted in Figure 1: Was the toxicant going to lead to extinction of the population? In the case of *C. elegans*, under the conditions of our experiment this was not likely to happen, because when exposed to cadmium chloride, fitness decreased during the first generation but increased slightly, but significantly, after consecutive generations (chapter 5). This means that *C. elegans* was able to partly oppose the negative effect of cadmium chloride on its fitness and therefore would be able to maintain a substantial rate of population increase. It was shown that the maximum fitness of *C. elegans* depended strongly on the trade-off between sperm maturation time and juvenile development. Cadmium chloride decreased the fitness of *C. elegans* by impairment of juvenile development. The sperm maturation time remained constant. After long-term exposure of multiple generations, *C. elegans* counteracted the effect on juveniles by growing faster and increasing reproduction and fitness. This illustrates that the combination of detailed knowledge of the life-cycle and life-history modelling provides insight into the underlying mechanisms of toxicant induced life-cycle changes and fitness consequences.

At low copper concentrations (till 20 µM), the fitness of *P. acuminatus* increased, only at higher copper concentrations (60 µM) it decreased (Kammenga and Riksen, 1996; see Table 1). This implies that, in spite of sublethal effects, low copper concentrations did not lead to a toxicological impairment at a high organisational level of *P. acuminatus*. This underlines the fact that biomarker responses of effect should correlate to other toxicological endpoints, preferably at higher organisational levels, and that the ecological relevance of the toxicological endpoints should be taken into account when risk assessment was involved. For risk assessment, it is very important not to rely on only one species, because the sensitivity of different species to environmental contamination might differ substantially. Therefore, always several species, preferably belonging to different trophic levels, should be incorporated in any risk assessment protocol (Kammenga *et al.*, 2000). Furthermore, the fitness consequences of cadmium were only measured for one generation of *P. acuminatus*. The possible impact of cadmium on fitness after consecutive generations might reveal a different toxicological outcome of the same parameter.

6.3 Hsp60 response curves in general

When the hsp60 response in *P. acuminatus* after 24 hours of exposure to increasing zinc chloride concentrations (chapter 3) was compared to the hsp60 response reported in other papers in other organisms, it appeared that similar response curves were obtained. This was independent of exposure to an increasing range of a stress factor or to one steady-state stress factor, but the response measured at different time intervals. The hsp60 response increased until a maximum was reached and then decreased until finally control levels were obtained (and sometimes even lower levels than control levels). The maximum value and the width of

the response curve depended upon many factors, e.g. the organism and organism tissue involved, the stress factor, the level upon which the response was measured (mRNA or protein level). A general theoretical hsp60 response course is depicted in Figure 2. The curves 1, 2 and 3 could represent three different levels (e.g. concentrations) of a stress factor with curve 1 being the highest and curve 3 being the lowest level of the stress factor. In this case, the response was measured in time (x-axis). Time could represent exposure time or time after exposure, depending upon the experimental design and the reaction time of the response. Alternatively, the curves 1, 2 and 3 could also represent three different time points at which the response was measured with the increasing stress factor level as variable (x-axis).

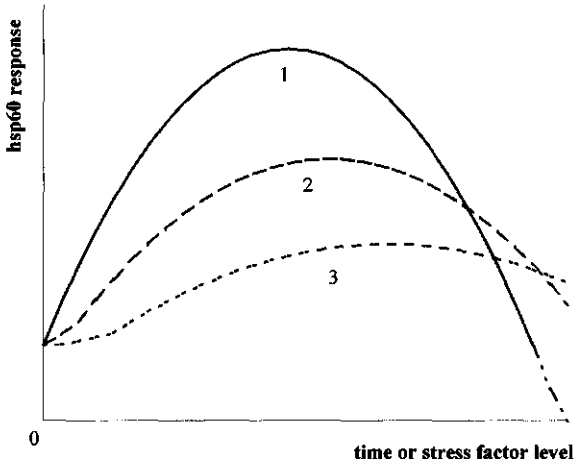

Figure 2: General theoretical hsp60 response curves. The y-axis indicates the relative hsp60 response (as percentage of the control), the curves 1-3 the response at three stress factor levels with the x-axis being the time. Alternatively, the curves 1-3 represent the response at three time points with the x-axis being the increasing stress factor level.

An optimum response curve implies that one data point from the y-axis corresponds to two points on the x-axis, unless the measured point is the maximum of the curve. This means that, even when the response curve is known, the hsp60 response data (y-axis) cannot unambiguously be related to the concomitant stress level (x-axis). Therefore, the use of other biomarkers is required to reveal which part of the hsp response curve is involved.

6.4 Regulation of the hsp(60) response

When discussing the hsp60 response in *P. acuminatus* at the mRNA and protein level (chapter 3), the question emerges whether the response would be regulated transcriptionally, translationally, or both. Generally, heat shock gene expression is controlled both transcriptionally and translationally (Panniers, 1994), though depending upon the organism and cell type within an organism, the emphasis can differ (Lindquist, 1986). In *Escherichia coli* (Yamamori and Yura, 1980) and yeast (McAlister and Finkelstein, 1980; Lindquist, 1981), the heat shock response was controlled primarily at the level of transcription. In *Drosophila melanogaster* regulation was exerted more equally on both transcription and translation (e.g. Storti *et al.*, 1980; Lindquist, 1981). In *Xenopus laevis*, the response of

somatic cells was controlled primarily at the transcriptional level, whereas the response of oocytes was regulated mainly at the translational level (Bienz and Gurdon, 1982). These differences make biological sense. They are depending for example upon the half-life of the mRNA, the velocity of the change in protein synthesis required upon stress exposure and the efficiency of mRNA and protein synthesis (Lindquist, 1986), which might differ between organisms and even between cell types within an organism. Snutch and Baillie (1983) suggested that the heat shock response in the nematode *C. elegans* was under both transcriptional and translational control, as evidenced from *in vivo* and *in vitro* polypeptide expression patterns.

In many papers describing the hsp60 response at the mRNA and protein level upon stress exposure (see chapter 3), the hsp60 mRNA level was reported to increase prior to or simultaneously with an increase at the hsp60 protein level independent of the organism or organism tissue under investigation. This implies that transcriptional regulation is involved in hsp60 expression. The hsp60 protein level remained usually elevated long after quenching of the hsp60 mRNA response (see chapter 3). Besides the probably substantial differences between mRNA and protein synthesis and degradation process rates, this might indicate that post-transcriptional and/or translational control could be important in the regulation of hsp60 expression as well. The hsp60 response in *P. acuminatus* would also be regulated both transcriptionally and translationally, but where the emphasis is remains unknown. From the data obtained in this thesis no conclusions can be drawn concerning this issue. Maybe there was an elevated hsp60 mRNA response before an increase at the protein level was measured, but even if so, transcriptional regulation would be important but presumably not the only control mechanism.

In both transcription and translation many factors are involved that all can exert a degree of control on these processes (e.g. Panniers, 1994; Morimoto *et al.*, 1992; Lis and Wu, 1993). This control is very complex. Besides translational control, often also post-transcriptional control is discerned when discussing regulation of hsp expression. For instance, it has been suggested that the continued synthesis of hsps was dependent not upon continued transcription of *hsp* genes, but upon continued translation of stable hsp messages (Yost *et al.*, 1990). These hsp mRNAs were degraded during recovery with a timing that varied dramatically with the severity of the preceding heat shock. Furthermore, hsp70 mRNAs, which were extremely unstable at normal temperatures, were stabilized by heat shock (Theodorakis and Morimoto, 1987; Lindquist and Petersen, 1990). Except for the mRNA half-life, also the splicing of the messages could be important in the post-transcriptional control. Severe heat shock blocked the splicing of intervening sequences from mRNA precursors (Yost and Lindquist, 1988). Most of the heat shock genes that had been cloned and sequenced did not contain any introns, allowing their messages to circumvent this block (Lindquist, 1986).

However, the partial *hsp60* gene sequence elucidated in *P. acuminatus* (EMBL/genbank libraries, accession number AJ130947) contained several introns, as well as many other *hsp* genes (e.g. Rebbe *et al.*, 1989; Takahashi *et al.*, 1992; Sconzo *et al.*, 1992; Hosokawa *et al.*, 1993; Uoshima *et al.*, 1993; Das *et al.*, 1997). In our case, the occurring introns in the *hsp60* gene might be explained by the fact that hsp60 is a constitutively expressed protein, also abundant at normal temperatures. The heat-induced genes do not contain any intervening sequences, but those that are expressed at normal temperatures do (Dworniczk and Mirault, 1987). So, it is possible that the partial *hsp60* gene sequence encoded the hsp60 isoform, which is designated in the two dimensional immunoblots of *P. acuminatus* described in chapter 2 as spot number 7 (Fig. 1D). This spot was present at all tested temperatures (see Figs 1D, 1E and 1F, chapter 2) and was (nearly) not upregulated by heat-shock. In contrast,

spot 9 and 10 were not present at the control temperature (Fig. 1D, chapter 2), but were strongly induced by heat shock (see Figs 1E and 1F, chapter 2), so possibly there were no introns present in their corresponding genes. This implies that maybe hsp60 isoforms exist that are strictly heat-inducible. That spot 7 was present even after heat-shock could be explained by the fact that, besides differences in synthesis and degradation rates, the splicing of essential heat shock transcripts might be somewhat more resistant to heat than the splicing of other transcripts (Yost *et al.*, 1990). After metal exposure, two spots were detected and found to be increased (Fig. 2, chapter 2), resembling spot 9 and 10 of Figs 1E and 1F that were detected after heat shock (see chapter 2). Possibly, the protein bands of the one dimensional blots obtained after metal exposure of *P. acuminatus* in chapter 3 and 4 represented these hsp60 subunits/isoforms. This implies that the hsp60 mRNA sequence elucidated in *P. acuminatus* using primers based on the *hsp60* gene sequence (chapter 3) might be a different hsp60 isoform than the one(s) stained on the one dimensional protein blots. However, because hsp60 sequences are conserved (Lindquist and Craig, 1988), it is also possible that the primers recognized several hsp60 isoforms in *P. acuminatus* and therefore, the hsp60 mRNA response might also exist of a mixture of different hsp60 isoforms.

6.5 Heat shock protein responses: Suitable biomarkers for toxicant stress?

Heat shock protein responses are sensitive general stress indicators, so called tier I biomarkers (Sanders, 1990). Increased hsp levels indicate the presence of a stressor, but the nature of the stressor can usually not be extracted from the hsp response. This means that the response to a stressor can often not be distinguished from the response to a confounding factor. A monitoring strategy using biomarkers would be most effective if tier I biomarkers would be used for initial screening, combined with tier II biomarkers (specific responses) to help identify the stressor(s) (Sanders, 1990). This implicates that not only the hsp response, but also other biomarkers should always be used in combination. This has also been advocated by Kammenga *et al.* (2000).

The usefulness of hsps as biomarkers was demonstrated, but questioned as well (De Pomerai, 1996; Pyza, 1997; Bierkens, 2000). It has been argued that different agents could induce different families of hsps, so not every hsp family responded to every type of stressor. Furthermore, different isoforms within the hsp families might respond differently to different stressors (this thesis). The variability of the hsp response of replicates was often very high, hampering the detection of any statistically significant differences and making it necessary to use many replicates (see chapter 3 and 4). This could make the application of hsps as biomarkers less favorable as easy-to-use, quick and cheap tools.

From this thesis it can be concluded that the hsp60 response in *P. acuminatus* could be used as a biomarker for metal exposure, but with the following limitations:

1. An increase of the hsp60 response in the nematode indicates the presence of a proteotoxic stress factor. To identify this stress factor, additional measurements would have to be performed, e.g. chemical analyses, the application of tier II biomarkers.
2. The hsp60 response in *P. acuminatus* is only elevated within a restricted and relatively low concentration range of the metal(s). No elevation of the response means that the stressor level is either too low or too high. The hsp60 response should always be related to other biomarker responses in order to interpret the response. This is not only important in case no elevation is measured, but also to determine where the hsp60 response should

be located compared to the maximum induction value, indicating whether the response is increasing or already quenching.

3. Enough replicates should be used to make sure that any statistically significant differences are not masked by the high variability of the individual measurements.

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Summary

Terrestrial invertebrates offer meaningful targets for assessing the potential adverse effects of chemicals on soil ecosystems. Invertebrates play a major role in the functioning of the soil ecosystem by enhancing the soil structure, mineralization and the decomposition of organic material, and because of their role in the foodweb. The most dominant group of terrestrial invertebrates, in fact of all multicellular organisms on earth, are nematodes, also called threadworms or roundworms. Nematodes are usually small (0.2-2 mm), transparent and present in almost every habitat on earth. In soil, they usually occur in high abundances and the nematode community comprises a considerable species diversity (Sohlenius, 1980). Nematodes belonging to the terrestrial bacterial feeders inhabit the interstitual water of soil particles (Houx and Aben, 1993). Therefore, they are subjected directly to the dissolved fraction of contaminants in soils, apart from being subjected indirectly via the foodsource. After extraction from the soil, many of these terrestrial bacterivorous nematodes can easily be reared in the laboratory in growth media or on agar plates with bacteria as foodsource.

Plectus acuminatus (Nematoda, Torquentia, Plectidae) Bastian 1865 is an example of such a free-living terrestrial bacterivorous nematode species, easy to rear in the laboratory. This species has an egg-to-egg period of approximately 3 weeks and a life span of about 3 months at 20°C. *P. acuminatus* appeared to be a suitable species for toxicity tests (Kammenga *et al.*, 1996) and is ubiquitous in the moderate regions of the world (e.g. in soils in the Netherlands (Bongers, 1988) and the UK (Arts, unpublished)).

Caenorhabditis elegans (Nematoda, Secernentea, Rhabditidae) Maupas 1899 is another example of a free-living terrestrial bacterivorous nematode species. It is the most investigated nematode species in laboratory experiments that exists. *C. elegans* strain N2 has originally been extracted from soil from the area of Bristol, UK, but has not been found in Dutch soils yet. It has been reared and maintained in the laboratory for decades. *C. elegans* is homozygous and doesn't suffer from any inbreed depression. Its life-cycle is very short with an egg-to-egg period of nearly 3 days and a total life span of about 20 days at 20°C (Wood, 1988). Therefore, this nematode species is very suitable to study life-cycle traits and to perform multi-generation experiments.

The purpose of this thesis is to evaluate metal stress in free-living terrestrial bacterivorous nematodes by measuring the response on the one hand at a very low organisational level (biomarker response) and on the other hand at a high organisational level (fitness consequences at the population level after consecutive generations). The biomarker response can predict effects of toxicants on cellular function, which might lead to changes in the physiology and/or histology of an organism. These changes possibly exert effects on life-cycle traits, which could lead to changes in fitness, which might eventually lead to extinction of the population.

Biomarkers are changes at the molecular, biochemical or cellular level in organisms following exposure to pollutants (Peakall and Shugart, 1992; Deplegde and Fossi, 1994) and are usually the first detectable responses to environmental perturbation. Because these alterations underlie all effects at higher organisational levels, they can be helpful tools in ecotoxicological risk assessment.

If we select as biomarker cellular and biochemical events which are intimately involved in protecting and defending the cell from environmental insults, we have ideal candidates for biomarkers of exposure and possibly of effect (Sanders, 1990). Cells dramatically alter their gene expression in response to environmental stress, attempting to protect themselves from damage and to repair existing damage (Schlesinger *et al.*, 1982). This response is called the cellular stress response. Changes in gene expression associated with the stress response are extremely rapid and result in the induced synthesis and accumulation of stress proteins. One group of stress proteins are the heat shock proteins (hsps), first discovered upon heat exposure but later found to be induced by a wide variety of chemical, physical and biological stressors (e.g. listed in Nover, 1991 and Sanders, 1993). Hsps possibly all function as molecular chaperones (Ellis, 1987), for one, facilitating the synthesis, folding, assembly and intracellular transport of many proteins, reducing protein denaturation and aggregation and aiding in protein renaturation (e.g. Ellis and van der Vies, 1991; Parsell and Lindquist, 1993). The common signal elicited by all hsp-inducing stressors involves an abnormally high concentration of damaged/aggregated proteins within cells, a phenomenon generally referred to as 'proteotoxicity' (Hightower, 1993). Hsp biomarkers give an integrated response summarizing the total proteotoxic damage caused within the target organism or organism tissue.

Each hsp is the member of a multigene family, regulated by different promotors and coding for closely related protein isoforms (Lindquist, 1986). Based on their molecular weight, hsps can be classified into different families (Sanders, 1993). The family of 55-65 kDa is called chaperonin. The members of this family have thus far been found in eubacteria and in eukaryotic cells, almost exclusively in organelles which are probably of endosymbiotic origin (mitochondria, chloroplasts) (Hemmingsen *et al.*, 1988) designated hsp60, stress-60, cpn60, GroEL (*E. coli*) or RuSBP (Rubisco Subunit Binding Protein (chloroplast)). Hsp60 is a nucleus-encoded, constitutively expressed protein. Under stressfull conditions, the hsp60 expression can be dramatically increased. Together with the ubiquitous hsp70 family, which is the most highly conserved and the largest of all the hsp families, the hsp60 family has great potential as a biomarker for general stress (Sanders, 1990).

Therefore, both the hsp70 and hsp60 response were qualitatively analyzed in the nematode *P. acuminatus* (see chapter 2) in order to select the most sensitive hsp-biomarker to increasing metal concentrations. The hsp70 and hsp60 responses were studied following exposure to heat, to copper chloride and to cadmium chloride. Mini two-dimensional polyacrylamide gel electrophoresis was used for protein separation. Poly- and monoclonal antibodies raised against hsp70 or hsp60 in various organisms were used to detect the respective hsps by immunoblotting. Both hsp60 and hsp70 could be identified after exposure of the nematodes to heat, indicating the broad cross reactivity among species to the antibodies used. The induction of hsp60 in *P. acuminatus* was related to increased concentrations of cadmium and copper chloride. For copper chloride, the induction of hsp60 was 3 orders of magnitude more sensitive than was the EC20 for reproduction; for cadmium chloride, the hsp60 induction was 2 orders of magnitude more sensitive. The hsp70 response in *P. acuminatus* was also elevated after exposure of the nematodes to cadmium and copper chloride, but this response was relatively weak compared to the hsp60 response. Therefore, it was concluded that the hsp60 response may be suitable as a potential biomarker to metal stress in *P. acuminatus*.

The hsp60 response in *P. acuminatus* has been further investigated quantitatively in the laboratory, at the protein level as well as at the mRNA level after exposure to various metals (see chapter 3). The mRNA response may be more sensitive and reproducible compared to the protein response and was therefore worth considering. Both the hsp60 protein and mRNA

response were measured after 24 hours of exposure to either zinc chloride (0-550 µM) or copper chloride (0-59 µM), the protein response also after 24 hours of exposure to cadmium chloride (0-109 µM). Furthermore, we identified hsp60 in *P. acuminatus* by elucidating its full-length mRNA sequence and deduced amino acid translation and comparing this to other known sequences. After exposure of the nematodes to zinc chloride, a significant optimum curve was found for the hsp60 response at the protein level, with a maximum induction of over 8 fold the control response at a concentration of 291 µM zinc chloride. Most likely, the hsp60 response increased until the ability of the heat shock system to react to increasing metal concentrations reached its climax, after which a further increase in metal concentrations resulted in a decline of the hsp60 level, which might be interpreted as a result of pathological tissue damage as described by Eckwert *et al.* (1997) concerning the hsp70 response in the isopod species *Oniscus asellus*. A significant hsp60 increase at the protein level was also detected with increasing copper chloride concentrations, but the maximum hsp60 induction was not reached within the investigated copper concentration range. When the nematodes were exposed to cadmium chloride, no significant trend was observed. At the mRNA level, in *P. acuminatus* no considerable hsp60 induction was obtained when compared to control levels and to the protein levels at the investigated metal concentration range after 24 hours of exposure. Though the variability at the hsp60 protein level in *P. acuminatus* was much higher compared to the hsp60 mRNA level, the increase upon metal exposure was much higher at the protein level and occurred at higher metal concentrations. Therefore, the hsp60 protein response in *P. acuminatus* may have more potential as a biomarker for metal stress than the hsp60 mRNA response.

In chapter 4, the application of the hsp60 protein response in *P. acuminatus* as a biomarker for metal pollution is evaluated in an in situ bio-assay in a field experiment along a metal gradient near Avonmouth, UK. Because it is impossible to determine nematodes to the species level without killing or at least heavily stressing them, *P. acuminatus* specimens were transplanted into six field sites along the metal gradient and the hsp60 protein response was measured. The response appeared to be significantly higher in the nematodes transplanted into the field site with the lowest metal concentrations compared to the other field sites. The responses of the nematodes in the other field sites did not significantly differ from each other. It can be concluded that the hsp60 response in *P. acuminatus* alone was not a suitable biomarker for heavily contaminated soils. However, this biomarker had indicative value when related to other biomarker responses measured simultaneously in the same field sites (e.g. the hsp70 response in the isopod species *O. asellus* and *Porcellio scaber*). Furthermore, it might be a suitable biomarker for less heavily contaminated soils. This would have to be investigated in field experiments, because laboratory experiments provide no alternative.

From this thesis it can be concluded that the hsp60 response in *P. acuminatus* could be used as a biomarker for metal exposure, but with the following limitations:

1. An increase of the hsp60 response in the nematode indicates the presence of a proteotoxic stress factor. To identify this stress factor, additional measurements would have to be performed, e.g. chemical analyses, the application of biomarkers which identify specific stressors.
2. The hsp60 response in *P. acuminatus* is only elevated within a restricted and relatively low concentration range of the metal(s). No elevation of the response means that the stressor level is either too low or too high. The hsp60 response should always be related to other biomarker responses in order to interpret the response. This is not only important in case no elevation is measured, but also to determine where the hsp60 response should be located compared to the maximum induction value, indicating whether the response is increasing or already quenching.

The outcome of short-term toxicity studies, such as the hsp responses described in this thesis, may not be used for predicting long-term demographic effects. Because effects at the population level are mediated through effects on fitness, the change in fitness under metal stress in a multi-generation experiment is studied using the nematode *C. elegans* (chapter 5). The strong advantage of testing multiple generations instead of one generation is the detection of possible trade-off mechanisms among life-history traits and fitness consequences, thus eliciting the probable course of the final consequences of chronic metal stress on the existence of the population. A life-history model of *C. elegans* was developed to calculate fitness maximisation in populations exposed to cadmium chloride during multiple generations. It was shown that the maximum fitness of *C. elegans* depended strongly on the trade-off between sperm maturation time and juvenile development. Once *C. elegans* was exposed to cadmium chloride, fitness decreased during the first generation. After exposure of consecutive generations, fitness increased slightly but significantly compared to the first exposed generation, while various life-cycle traits were strongly affected. The life-history modelling of *C. elegans* showed that cadmium chloride decreased fitness by impairment of juvenile development. The sperm maturation time remained constant. After long-term exposure of multiple generations, *C. elegans* counteracted the effect on juveniles by growing faster and increasing reproduction and fitness. This chapter illustrates that the combination of detailed knowledge of the life-cycle and life-history modelling provides insight into the underlying mechanisms of toxicant induced life-cycle changes and fitness consequences.

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Samenvatting

Terrestrische evertebraten vormen een waardevolle doelgroep voor het inschatten van potentiële nadelige effecten van chemicaliën op bodemecosystemen. Evertebraten spelen een hoofdrol in het functioneren van het bodemecosysteem doordat ze de bodemstructuur verbeteren en de afbraak van organisch materiaal en de mineralisatie bevorderen. De relatief grootste groep van terrestrische evertebraten, in feite van alle meercellige organismen op aarde, zijn nematoden, ook wel draadwormen of rondwormen genoemd. Nematoden zijn meestal klein (0.2-2 mm), doorzichtig en aanwezig in vrijwel iedere habitat op aarde. In de bodem komen ze gewoonlijk voor in grote aantallen en de nematodengemeenschap heeft een hoge soortendiversiteit (Sohlenius, 1980). Nematoden die behoren tot de terrestrische bacterie-eters leven in de waterfilm die de bodemdeeltjes omgeeft (Houx and Aben, 1993). Ze worden daardoor rechtstreeks blootgesteld aan de opgeloste fractie van milieuverontreinigende stoffen in bodems, naast indirecte blootstelling via het voedsel. Vele van deze terrestrische, bacterie-etende nematoden kunnen na extractie uit de bodem gemakkelijk gekweekt worden in het laboratorium in groeimedia of op agar platen, met bacteriën als voedselbron.

Plectus acuminatus (Nematoda, Torquentia, Plectidae) Bastian 1865 is een voorbeeld van zo'n vrijlevende, terrestrische, bacterie-etende nematodesoort, die gemakkelijk in het laboratorium kan worden gekweekt. Bij deze soort duurt de ei-tot-ei periode bij 20°C ongeveer 3 weken en de totale levensduur is ongeveer 3 maanden. *P. acuminatus* is geschikt gebleken voor toxiciteitstoetsen (Kammenga *et al.*, 1996) en komt algemeen voor in de gematigde streken (bijv. in de bodem in Nederland (Bongers, 1988) en Engeland (Arts, unpublished)).

Caenorhabditis elegans (Nematoda, Secernentea, Rhabditidae) Maupas 1899 is een ander voorbeeld van zo'n vrijlevende, terrestrische, bacterie-etende nematodesoort. Het is de best onderzochte nematodesoort in laboratoriumexperimenten die bestaat. *C. elegans* stam N2 is oorspronkelijk geëxtraheerd uit de grond in de omgeving van Bristol, Engeland, maar is nog niet gevonden in de Nederlandse bodem. Deze soort wordt al tientallen jaren gekweekt en in stand gehouden in het laboratorium. *C. elegans* is homozygoot en vertoont geen inteeltdepressie. De levenscyclus is erg kort: De ei-tot-ei periode duurt bij 20°C bijna 3 dagen en de totale levensduur is ongeveer 20 dagen (Wood, 1988). Hierdoor is *C. elegans* heel geschikt voor studies naar levenscyclusonderdelen en voor het uitvoeren van experimenten die meerdere generaties bestrijken (multi-generatie experimenten).

Het doel van dit proefschrift is het evalueren van metaalstress in vrijlevende, terrestrische, bacterie-etende nematoden door de respons enerzijds te meten op een heel laag organisatorisch niveau (biomarker respons) en anderzijds op een hoog organisatorisch niveau (fitness consequenties op het populatieniveau na opeenvolgende generaties). De biomarker respons kan effecten van toxicanten op het cellulaire functioneren voorspellen, wat mogelijk kan leiden tot veranderingen in de fysiologie en/of histologie van een organisme. Deze veranderingen zouden effect kunnen sorteren op levenscyclusonderdelen, welke zouden kunnen leiden tot veranderingen in fitness, wat mogelijk uiteindelijk kan leiden tot het uitsterven van de populatie.

Biomarkers zijn veranderingen op het moleculaire, biochemische of cellulaire niveau in organismen na blootstelling aan verontreinigende stoffen (Peakall and Shugart, 1992; Deplegde and Fossi, 1994) en zijn gewoonlijk de eerste detecteerbare reacties op verstoring van het milieu. Omdat deze veranderingen ten grondslag liggen aan alle effecten op hogere organisatorische niveaus, kunnen ze behulpzaam zijn in de ecotoxicologische risicio-analyses.

Als we als biomarker cellulaire en biochemische reacties nemen die nauw betrokken zijn bij de bescherming en verdediging van de cel tegen aanvallen vanuit de omgeving, hebben we ideale kandidaten voor biomarkers van blootstelling en mogelijkerwijze effect (Sanders, 1990). Cellen veranderen hun genexpressie ingrijpend als reactie op omgevingsstress, waarbij ze zichzelf trachten te beschermen tegen schade en ontstane schade pogen te repareren (Schlesinger *et al.*, 1982). Deze reactie wordt de cellulaire stressrespons genoemd. Veranderingen in genexpressie geassocieerd met de stressrespons zijn razendsnel en resulteren in de geïnduceerde synthese en accumulatie van stresseiwitten. Een groep van stresseiwitten zijn de heat shock ('hitteschok') eiwitten (hsps). Ze zijn ontdekt na blootstelling van organismen aan verhoogde temperaturen, maar bleken later ook te worden geïnduceerd door een grote variëteit aan chemische, fysische en biologische stressfactoren (bijv. beschreven door Nover, 1991 en Sanders, 1993). Hsps functioneren mogelijk allemaal als moleculaire chaperons ('begeleiders') (Ellis, 1987), door onder meer de synthese, vouwing, assemblage en het intracellulaire transport van vele eiwitten te vergemakkelijken, eiwitdenaturatie en -samenklontering tegen te gaan en the helpen bij eiwitrenaturatie (bijv. Ellis and van der Vies, 1991; Parsell and Lindquist, 1993). Het algemene signaal, opgewekt door alle hsp-inducerende stressfactoren, is een abnormaal hoge concentratie van beschadigde/samengeklonterde eiwitten in cellen, een fenomeen wat algemeen bekend staat als 'proteotoxiciteit' (Hightower, 1993). Hsp-biomarkers geven een geïntegreerde respons van de totale proteotoxische schade in een doelorganisme of doelorgaan.

Iedere hsp is lid van een multigene familie, gereguleerd door verschillende promotoren en coderend voor nauw verwante eiwit-isovormen (Lindquist, 1986). Hsps kunnen ingedeeld worden in verschillende families, gebaseerd op hun moleculaire gewicht (Sanders, 1993). De familie van 55-65 kDa wordt chaperonin genoemd. De leden van deze familie zijn tot nu toe gevonden in eubacteria en in eukaryotische cellen, bijna alleen in organellen die waarschijnlijk van endosymbiotische oorsprong zijn (mitochondriën, chloroplasten) (Hemmingsen *et al.*, 1988). Er wordt naar gerefereerd als hsp60, stress-60, cpn60, GroEL (*E. coli*) of RuSBP (Rubisco Subunit Binding Protein (chloroplast)). Hsp60 is een nucleus-gecodeerd, constitutief tot expressie gebracht eiwit. Onder stressvolle omstandigheden kan de hsp60-expressie spectaculair toenemen. Samen met de alomtegenwoordige hsp70 familie, die de meest geconserveerde en grootste hsp familie is van allemaal, heeft de hsp60 familie grote potentie als biomarker voor algemene stress (Sanders, 1990).

Om deze reden zijn zowel de hsp70- als de hsp60-respons kwalitatief geanalyseerd in de nematode *P. acuminatus* (zie hoofdstuk 2), met als einddoel de meest gevoelige hsp-biomarker te selecteren voor oplopende metaalconcentraties. De hsp70- en hsp60-respons werden onderzocht na blootstelling aan verhoogde temperatuur, aan koperchloride en aan cadmiumchloride. Mini twee-dimensionale polyacrylamide gelelectrophorese werd gebruikt om de eiwitten te scheiden. Poly- en monoclonale antilichamen, opgewekt in verschillende organismen tegen hsp70 of hsp60, werden gebruikt om de betreffende hsps te detecteren met immunoblotten. Zowel hsp60 als hsp70 konden geïdentificeerd worden na blootstelling van de nematoden aan verhoogde temperatuur, wat duidt op de brede kruisreactiviteit van de gebruikte antilichamen tussen verschillende organismen. De hsp60-inductie in *P. acuminatus* was gerelateerd aan toegenomen concentraties cadmium- en koperchloride. De hsp60-

inductie was bij koperchloride 3 ordes van grootte en bij cadmiumchloride 2 ordes van grootte gevoeliger dan de EC20 voor reproductie. De hsp70-respons in *P. acuminatus* was ook toegenomen na blootstelling van de nematoden aan cadmium- en koperchloride, maar deze respons was relatief zwak vergeleken met de hsp60-respons. Dit leidde tot de conclusie dat de hsp60-respons geschikt zou kunnen zijn als een potentiële biomarker voor metaalstress in *P. acuminatus*.

Vervolgens is de hsp60-respons in *P. acuminatus* op zowel het eiwit- als op het mRNA-niveau kwantitatief onderzocht in het laboratorium na blootstelling aan verschillende metalen (zie hoofdstuk 3). De mRNA-respons zou gevoeliger en reproduceerbaarder kunnen zijn vergeleken met de eiwit-respons en was daarom de moeite waard om in beschouwing te nemen. Zowel de hsp60-eiwit- als mRNA-respons werden gemeten na 24 uur blootstelling aan zinkchloride (0-550 µM) of aan koperchloride (0-59 µM), de eiwit-respons ook na 24 uur blootstelling aan cadmiumchloride (0-109 µM). Daarnaast hebben we hsp60 in *P. acuminatus* geïdentificeerd door de complete mRNA-sequentie en de daarvan afgeleide aminozuurvertaling op te helderen en dit te vergelijken met andere bekende sequenties. Na de nematoden blootgesteld te hebben aan zinkchloride werd een significante 'optimum curve' gevonden voor de hsp60-respons op het eiwitniveau, met een maximum inductie van meer dan 8 keer de respons van de controle, bij een concentratie van 291 µM zinkchloride. Zeer waarschijnlijk nam de hsp60-respons toe totdat het vermogen van het heat shock systeem om te reageren op toenemende metaalstress zijn hoogtepunt bereikte, waarna een verdere toename van de metaalconcentraties leidde tot een afname van het hsp60-niveau, wat misschien vertaald kan worden als het gevolg van pathologische weefselbeschadiging, zoals beschreven door Eckwert *et al.* (1997) betreffende de hsp70-respons in de pissebeddesoort *Oniscus asellus*. Een significante hsp60-toename op het eiwitniveau werd ook gevonden bij toenemende koperchlorideconcentraties, maar de maximum hsp60-inductie werd niet bereikt binnen de onderzochte koperconcentratiereeks. Na blootstelling van de nematoden aan cadmiumchloride werd geen significante trend waargenomen. Op het mRNA-niveau werd in *P. acuminatus* geen aanzienlijke hsp60-inductie verkregen vergeleken met de niveaus van de controle en vergeleken met de eiwitniveaus bij de onderzochte metaalconcentraties na 24 uur blootstelling. Hoewel de variabiliteit van de hsp60-eiwit-respons in *P. acuminatus* veel groter was dan die van de mRNA-respons, was de toename bij de eiwit-respons veel groter en trad op bij hogere metaalconcentraties. Daarom heeft de hsp60-eiwit-respons in *P. acuminatus* mogelijk meer potentie als biomarker voor metaalstress dan de hsp60-mRNA-respons.

In hoofdstuk 4 wordt de toepassing van de hsp60-eiwit-respons in *P. acuminatus* als biomarker voor metaalverontreiniging geëvalueerd in een in situ bio-assay in een veldexperiment langs een metaalgradiënt bij Avonmouth, Engeland. Omdat het onmogelijk is om nematoden te determineren tot op de soort zonder ze te doden of zonder tenminste hevige stress te veroorzaken, werden *P. acuminatus* exemplaren getransplanteerd in zes veldlocaties langs de metaalgradiënt en werd de hsp60-eiwit-respons gemeten. De respons bleek significant hoger te zijn in de nematoden die getransplanteerd waren in de veldlocatie met de laagste metaalconcentraties vergeleken met de andere veldlocaties. De respons van de nematoden in de andere veldlocaties waren niet significant verschillend van elkaar. Geconcludeerd kan worden dat de hsp60-respons in *P. acuminatus* op zichzelf geen geschikte biomarker was voor zwaar verontreinigde bodems. Deze biomarker had echter indicatieve waarde wanneer ze gerelateerd werd aan andere biomarkers, die gelijktijdig gemeten werden in dezelfde veldlocaties (bijv. de hsp70-respons in de pissebeddesoorten *O. asellus* en *Porcellio scaber*). Bovendien zou de hsp60-respons in *P. acuminatus* een geschikte biomarker kunnen zijn voor minder zwaar verontreinigde bodems. Dit zou onderzocht

moeten worden in veldexperimenten, omdat laboratoriumexperimenten geen alternatief bieden.

Uit dit proefschrift kan geconcludeerd worden dat de hsp60-respons in *P. acuminatus* gebruikt kan worden als biomarker voor metaalblootstelling met de volgende restricties:

1. Een toename van de hsp60-respons in de nematode duidt op de aanwezigheid van een proteotoxische stressfactor. Om deze stressfactor te identificeren zouden additionele metingen verricht moeten worden, bijv. chemische analyses, de toepassing van biomarkers die specifieke stressfactoren identificeren, etc..
2. De hsp60-respons in *P. acuminatus* neemt slechts toe binnen een beperkt en relatief laag concentratiebereik van de metalen. Geen toename van de respons betekent dat het stressniveau ofwel te laag is, ofwel te hoog. De hsp60-respons zou altijd gerelateerd moeten worden aan andere biomarkers om de respons te kunnen interpreteren. Dit is niet alleen belangrijk wanneer geen toename wordt gemeten, maar ook om te bepalen waar de hsp60-respons geplaatst moet worden ten opzichte van de maximale inductiewaarde, zodat bekend wordt of er sprake is van een toenemende of juist al uitdovende respons.

De uitkomst van korte termijn toxiciteitsstudies, zoals de hsp-respons beschreven in dit proefschrift, kunnen niet gebruikt worden om demografische effecten op de lange termijn te voorspellen. Omdat effecten op het populatieniveau tot stand komen door effecten op fitness, werd de fitnessverandering onder metaalstress in een multi-generatie experiment onderzocht bij de nematode *C. elegans* (hoofdstuk 5). Het grote voordeel van het testen van meerdere generaties in plaats van één generatie is de detectie van mogelijke trade-off (='ruil')mechanismen tussen levenscyclusonderdelen en fitness consequenties, waardoor het mogelijke verloop van de uiteindelijke consequenties van chronische metaalstress op het voortbestaan van de populatie opgehelderd wordt. Er werd een levensgeschiedenismodel van *C. elegans* ontwikkeld om de maximalisatie van fitness te berekenen in populaties die blootgesteld zijn aan cadmiumchloride gedurende meerdere generaties. Er werd aangetoond dat de maximale fitness van *C. elegans* sterk afhing van de trade-off tussen de spermarijpingstijd en de juveniele ontwikkeling. Wanneer *C. elegans* eenmaal blootgesteld werd aan cadmiumchloride nam de fitness af gedurende de eerste generatie. Na opeenvolgende blootgestelde generaties nam de fitness weer in geringe mate, maar significant, toe vergeleken met de eerste blootgestelde generatie, terwijl verschillende levenscyclusonderdelen sterk werden beïnvloed. De levensgeschiedenismodellering van *C. elegans* toonde aan dat cadmiumchloride de fitness deed afnemen door de verslechtering van de juveniele ontwikkeling. De spermarijpingstijd bleef constant. Na blootstelling van meerdere generaties werd het effect op juvenielen gedeeltelijk gecompenseerd door snellere groei en een toename van de reproductie en fitness. Dit hoofdstuk laat zien dat de combinatie van gedetailleerde kennis van de levenscyclus en levensgeschiedenismodellering inzicht kan verschaffen in de onderliggende mechanismen van de door een toxicant geïnduceerde levenscyclusveranderingen en fitness consequenties.

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Dankwoord

Toen ik in september 1996 op de leerstoelgroep Nematologie met mijn promotieonderzoek begon, had ik verschillende korter lopende en minder fundamentele onderzoeken achter de rug. Het leek me nu heerlijk om eens 4 jaar aan één onderzoek te werken, dan had ik eindelijk eens tijd om alles goed uit te diepen, dacht ik. Die 4 jaar zijn voorbij gevlogen lijkt het wel en tijd voor verdieping was er zeker wel, maar uiteindelijk heeft alles zijn beperkingen. Zoals bijna elk promotieonderzoek kende ook dat van mij zijn ups en downs. Het was een hele enerverende periode, waarin ik ontzettend veel geleerd heb. Dat dit proefschrift nu voltooid is, had ik alleen nooit voor elkaar gekregen, vandaar dat ik hierbij graag van de gelegenheid gebruik maak om een aantal mensen te bedanken.

In de eerste plaats mijn begeleider en co-promotor Jan Kammenga, die mij altijd met raad en daad terzijde stond en ervoor gezorgd heeft dat mijn aio-periode op de juiste manier volbracht is. Jan, ontzettend bedankt voor je onvoorwaardelijke steun en je vertrouwen in mij, voor je interesse, je kennis, je motiverende en stimulerende gesprekken, voor je inzet om mijn wetenschappelijke kennis en kennissenkring te verruimen.

Martijs Jonker, mijn collega-aio in de nematologie gecombineerd met toxicologie en kamergenoot, bedankt voor alles. De vruchtbare gesprekken, je toxicologische en statistische kennis, de SAS-uitwerkingen en zeker ook de gezelligheid heb ik erg kunnen waarderen. Geert Smant, bedankt voor je professionele hulp en je geduld om mij te begeleiden in het moleculaire werk en in de 2D electroforese. Charlotte van Twisk, jij ook bedankt, vooral voor dat laatste. Stephen Stürzenbaum, thanks for your fruitful professional work, for your support and for providing the opportunity for me to come over to Cardiff and perform the lightcycler experiments. I really appreciate everything you have done for me. Peter Kille, also thanks for your interest and support. Boudewijn van Veen, fantastisch zoveel als jij weet over beeldverwerking. Bedankt voor de scans, image analysing en de tips en interessante gesprekken.

To all BIOPRINT-II collegues and their assistants: Thanks for the good team work! I enjoyed the field work, the discussions and the good atmosphere. Jason, I admire your good organisational skills. Heinz, special thanks for your collaboration in the writing of our paper and thanks for your professional advices regarding my hsp work.

Mijn studenten Judith van Langevelde, Katrien Verkampen, Nina Liesker en Martijn Timmermans, jullie bedankt voor jullie inzet en jullie interesse in mijn onderzoek, Agnieszka Doroszuk, thanks for your help and interest. It was a great pleasure to work with you all. Jullie resultaten hebben bijgedragen aan de tot standkoming van dit proefschrift. Paul van Koert, Hein Overmars, Rikus Pomp, Jan Roosien, Hanny van Megen en Jan van Bezooijen, ik heb veel van jullie geleerd dankzij jullie praktische kennis, bekwaamheid en bereidheid om mij te helpen als ik hulp nodig had. Geweldig gewoon! Evert, bedankt voor de plaatjes. Lisette en je medewerksters, bedankt voor jullie hulp op het secretariaat. Iedereen op Nematologie en op het LMA, bedankt voor de goede werksfeer, voor de bereidheid om kennis te delen, om mee te denken en te helpen als dat nodig was, en natuurlijk, voor de gezelligheid. Dit alles is voor mij essentieel geweest om het aio-schap tot een goed einde te kunnen brengen. De grote flexibiliteit en collegialiteit op Nema/LMA werkte bijzonder

stimulerend. Everyone in the lab in Cardiff, thanks for your help and for the social events, you really made me feel welcome, I had a very good and fruitful time.

Als laatste, maar daarom zeker niet minder belangrijk, wil ik het thuisfront bedanken voor hun steun en voor mijn leven naast het aio-schap, waarin zich ook grote ontwikkelingen voordeden. Hein, bedankt voor je steun, geduld (jawel!) en relativeringsvermogen. Bram, je bent een fantastische knul! Mijn ouders en schoonouders wil ik speciaal bedanken voor hun steun en voor het passen op Bram, waardoor ik kon werken en mijn proefschrift binnen afzienbare tijd kon afronden.

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Curriculum vitae

Maria Silvestra José Hamelijnck-Arts werd geboren op 23 februari 1967 te Haps. In 1985 behaalde ze het VWO diploma aan het Merletcollege te Cuyk. Vervolgens ging ze Biologie studeren aan de Wageningen Universiteit (WU). Ze deed afstudeervakken op de leerstoelgroep Fytopathologie van de WU in samenwerking met het Plant Research International (PRI) te Wageningen en op de leerstoelgroep Virologie van de WU. Voor haar stage ging ze naar Costa Rica voor een epidemiologisch, fytopathologisch onderzoek. Na het behalen van haar bul in 1991 was ze tot 1996 – met enkele onderbrekingen - werkzaam op de sectie Virologie van de Plantenziektenkundige Dienst. Ze heeft daar verschillende onderzoeken gedaan betreffende virussen en viroïden in sier- en groentengewassen, vaak in samenwerking met de Nederlandse Algemene Keuringsdiensten. Daarnaast heeft ze nog enkele reizen begeleid voor SNP-natuurreizen. In september 1996 vervolgde ze haar wetenschappelijke loopbaan als assistent in opleiding aan de leerstoelgroep Nematologie van de WU met een onderzoek op het gebied van de toxicologie. De resultaten verkregen uit dit onderzoek zijn deels weergegeven in dit proefschrift.
Momenteel is ze aangesteld bij het bedrijf NOTOX te 's-Hertogenbosch, waar ze gaat werken als registratiemanager voor agro-chemicaliën en biociden.

The research described in this thesis was carried out at the Laboratory of Nematology, Wageningen University, Binnenhaven 10, 6709 PD Wageningen, The Netherlands, and was financially supported by the European Union, contract ENV4-CT96-0222.

Offset printed by: Ponsen & Looijen BV, Wageningen

Cover: The free-living, bacterivorous nematode species *Plectus acuminatus* and *Caenorhabditis elegans*