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Soils and Bioindicators – The Development of the Nematode *Steinernema feltiae* as a Bioindicator for Chromium VI Soil Pollution

STRIVE

Environmental Protection Agency Programme

2007-2013

SSTI Strategy for Science, Technology and Innovation

Comhshaol, Oidhreacht agus Rialtas Áitiúil
Environment, Heritage and Local Government

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EPA STRIVE Programme 2007–2013

Soils and Bioindicators – The Development of the Nematode *Steinernema feltiae* as a Bioindicator for Chromium VI Soil Pollution

(2008-FS-28-M1)

STRIVE Report

Prepared for the Environmental Protection Agency

by

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Table of Contents

Acknowledgements **ii**

Disclaimer **ii**

Details of Project Partners **iii**

Executive Summary **vii**

1 General Introduction **1**

1.1 Background 1

1.2 Project Aims 3

2 The Effects of Chromium VI on the Nematode *Steinernema feltiae* during *In Vivo* Development within Host Cadavers, and on the β-Tubulin Genes **4**

2.1 Introduction 4

2.2 Materials and Methods 4

2.3 Results 6

2.3 Discussion 8

2.4 Conclusions 14

3 The Effects of Chromium VI on the Infective Juvenile Stage of *Steinernema feltiae* in Contaminated Soils **16**

3.1 Introduction 16

3.2 Materials and Methods 16

3.3 Results 17

3.4 Discussion 24

3.5 Conclusions 27

4 Differential Gene Expression in *Steinernema feltiae* Nematodes in Response to Chromium VI Exposure in Contaminated Host Cadavers **29**

4.1 Introduction 29

4.2 Materials and Methods 29

4.3 Results 32

4.4 Discussion 36

5 General Discussion **38**

5.1 Conclusions 39

6 Relevance to Environmental Protection Agency Ireland Policy and Future Work **40**

References **41**

Acronyms and Abbreviations **47**

Executive Summary

This study set out to determine the suitability of the nematode *Steinernema feltiae* as a bioindicator for heavy metal pollution, specifically chromium VI. Hexavalent chromium (chromium VI) has been extensively shown to induce general environmental toxicity, as well as more specific effects of an acute and chronic nature, such as neurotoxicity, dermatoxicity, genotoxicity, carcinogeneticity, and immunotoxicity.

Nematodes have been studied as bioindicators for environmental stress for many years, and although great strides have been made in understanding the effects of contaminants on nematode communities and species, only one nematode to date has been officially described as a test organism. This nematode is *Caenorhabditis elegans*. There are strong ecological, biological and even practical arguments for the use of nematodes in assessing environmental disruptions, and the view is that the effects of pollution must be understood for as many species and genera as possible in order to inform environmental policy at effective levels.

Steinernema feltiae is ubiquitous in Ireland and can be easily isolated and identified. Its life cycle is completed in both external environmental habitats, such as soils, and within host cadavers. These characteristics present it as a viable alternative to other nematode test genera, in that it can be exposed to contaminants in a wider range of environmental habitats than exclusively soil-dwelling species such as *C. elegans*.

The investigation of the effects of chromium VI on *S. feltiae* was carried out using three principal approaches:

1. The effects of chromium VI on developing and feeding nematodes in vivo in host cadavers

2. The effects of chromium VI on the infective juvenile (IJ) stage as it lives in contaminated soils, and

3. The effects of chromium VI on the molecular stress response system of the nematode.

The first two approaches utilised microcosm-type experiments where fitness parameters such as progeny numbers (reproductive potential), development times, infectivity and survival rates were employed to observe the effects of the heavy metal on the nematode behaviour.

The final approach utilised polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing techniques to study the effects in the β-tubulin genes from nematodes exposed to chromium VI, and a differential gene expression technique (subtracted cDNA library generation) to identify genes regulated by chromium VI.

There were marked differences in the survival rates of *S. feltiae* between exposure to chromium VI during in vivo development and exposure to the IJs in soils. The nematode appeared to be considerably more resistant to chromium VI when it is present in the host cadavers than when exposed to it in soils at the IJ stage. Significantly higher numbers of offspring were recorded both from nematodes that were exposed to chromium VI as IJs in the soil prior to host infection, and from nematodes that developed in chromium VI-contaminated host cadavers. However, IJs exposed to chromium VI in soils exhibited significant reduction in infectivity, reduced survival rates, as well as increased development times. The results suggest the presence of a biological/physiological mechanism that allows the survivors of chromium VI exposure to actually increase progeny production in order to increase the chances of the population to survive the contamination event and preserve numbers.

The differential gene expression analysis generated a library of genes from *S. feltiae* putatively expressed in the presence of chromium VI. Six gene groups have so far been isolated, four of which demonstrated significant similarity to previously known genes. Most of these gene groups are known to be involved in stress response in other organisms. There were two gene groups which may be novel genes.

Studies and results described in this report considerably support the initial hypothesis that *S. feltiae* is a viable candidate for bioindicator development. It is relatively common in Ireland and can be isolated easily. It possesses a mechanism, or mechanisms, with which to potentially confer an advantage in a polluted environment. It also possesses a system comprising chromium VI-regulated genes that can be targeted in environmental assays. These molecular attributes, in conjunction with the fitness parameters described, provide a platform from which to understand the environmental effects of chromium VI in the environment.

This project was instrumental in demonstrating the good potential of *S. feltiae* in environmental bioindication for hexavalent chromium. Thus, subsequent studies are necessary to fully exploit this potential in order to lead to the development of specific environmental technologies utilising the nematode's behavioural traits and genes as suitable toxicity end points and biomarkers.

1 General Introduction

1.1 Background

The phylum Nematoda is a meiofaunal group that has colonised almost every conceivable habitat on the planet (Ruppert and Barnes, 1994). It has also evolved with great diversity and contains members that feed on every available food type. The majority of its members are microscopic and are strongly represented in terrestrial soils and benthic zones in marine and freshwater environments (Malakhov, 1994). As major components of the largely below-ground food web, they are consequently susceptible to disruptions. In recent years, this vulnerability has been exploited for *in situ* assessments of contamination and other environmental damage, using entire nematode community structures (Bongers and Ferris, 1999; Bakonyi et al., 2003; Nagy et al., 2004) and also using single nematode species (Black and Williams, 2001; Sochova et al., 2007; Harmon and Wyatt, 2008). On an even greater scale are laboratory investigations into the effects of individual and multiple components of contaminants on nematodes (Donkin and Dusenbery, 1994; Peredney and Williams, 2000; Boyd et al., 2003; Boyle and Kakouli-Duarte, 2008; Wang and Wang, 2008).

Although analysis of entire nematode community structures and populations is extremely important in environmental toxicological assessments as it allows us to understand the effects of disruptions on many levels of the soil ecosystem, the process can be labour intensive and requires considerable expertise. Due to the extended labour times required for this approach, studying nematode community structures may not be the best method to attempt to understand a pollution or contamination event when pressure from political, media or commercial sources is present. For this reason, single nematode candidates have been investigated far more widely and these studies have largely centred upon *Caenorhabditis elegans.* Its status as a widely investigated model organism, its ubiquity, and the fact that it can be easily cultured and stored have resulted in many studies demonstrating its usefulness as a test species for toxicity testing (Williams and Dusenbery, 1990; Hitchcock et al., 1997; Dhawan et al., 1999; Anderson et al., 2001; Boyd et al., 2001; Boyd and Williams, 2003a). Indeed, in the United States a guide was accepted that described the use of *C. elegans* in soil toxicity tests (American Society for Testing and Materials, 2002). An update of this guide has also recently been published (American Society for Testing and Materials, 2008). In these assays, the nematode is tested by measuring a range of lethal and sublethal effects, and the organism has proven to be sensitive to many different types of habitat disturbance, be they chemical, physical or biological. However, *C. elegans* is difficult to identify from environmental soils. This restricts ecological assessors to using laboratory-maintained strains and consequently potentially valuable information as to how the organism can survive disruption or contamination in an ecosystem is minimal or lacking.

There have been reports of nematode species exhibiting resistance, or actually doing better, after exposure to contaminants. Millward et al. (2001) described experiments where nematode populations actually increased, along with copepods and ostracods, probably due to the fact that these groups are largely particle feeders in sediment, as opposed to the deposit feeding habits of the other groups that were adversely affected by the contaminant exposure. Similarly, Jaworska and Gorczyca (2002) described the entomopathogenic nematode (EPN) *Steinernema feltiae* as benefiting from magnesium exposure prior to reproduction. Recently, exposure of chromium VI to this nematode during reproduction (i.e. within a suitable host insect) was shown to result in an increase in reproductive potential (Boyle and Kakouli-Duarte, 2008).

Why this occurs is currently unknown, although there are several possible explanations. Bongers and Ferris in a review (1999) stated that *C. elegans* (along with other members of the family Rhabditidae), being bacterivorous, flourishes in an environment of high microbial activity and is potentially surrounded by toxic microbial excretions, and therefore has become

relatively tolerant of pollutants when compared with other nematode genera and species. Other studies, such as that by Khanna et al. (1997), suggest that this tolerance is in terms of salinity, pH and water hardness. Indeed, the general consensus to date is that *C. elegans* is adversely affected by the presence of heavy metals (Dhawan et al., 2000; Anderson et al., 2001; Hoss et al., 2001; Boyd et al., 2003) and other types of contaminants such as ethanol (Dhawan et al., 1999), detergents (Harada et al., 2007) and man-made industrial chemicals such as dimethyl sulfoxide, bisphenol, aldicarb and benzophenone (Ura et al., 2002). Various approaches have been employed in these studies, including the use of transgenic bioluminescence strains (Lagido et al., 2001), online computer monitoring systems (Gerhardt et al., 2002), detection of ion–biomolecule interactions via atomic absorption spectrophotometry (AAS) (Tatara et al., 1997) and standard methods such as aquatic toxicity testing (Williams and Dusenbery, 1990) and aquatic acute toxicity testing (Ura et al., 2002). Although wide ranging, these methods are still heavily focused on one organism. If scientists are to pursue understanding of the effects of toxicants on the environment and the development of systems to observe and measure such effects, then it is imperative that additional nematode species are studied.

One such additional species is the EPN *S. feltiae*. This nematode is a ubiquitous species in Ireland (Griffin et al., 1991; Dillon et al., 1999) and can be easily isolated from soils (Bedding and Akhurst, 1975). It is a relatively small nematode and is an obligate and lethal parasite of insects (Reid and Hominick, 1992; Burnell and Stock, 2000). It is used as a biocontrol organism in horticulture and agriculture and its life cycle and physiology have been extensively studied. In the area of ecotoxicology, however, it has been largely ignored, with the exception of the study by Jaworska and Gorczyca (2002), in which the effects of metal ions on *S. feltiae* suggested a decrease in its ability to infect and reproduce in the wax moth *Galleria mellonella* after exposure to lead, cadmium, zinc and copper.

Entomopathogenic nematode fitness is a physiological barometer that may be tested using a number of biological characteristics as parameters. The ability to produce large numbers of progeny and the ability to infect host larvae effectively have been employed as fitness tests to determine the effects of age (Yoder et al., 2004), application method (Perez et al., 2004), temperature during development (Hazir et al., 2001), and production methods (Grewal et al., 1999) on different species of EPN. In all cases there were significant effects on most species. Additionally, traits such as infective juvenile (IJ) survival rates and progeny development times are also useful parameters with which to measure fitness. However, these EPN traits were rarely employed to detect the effects of contaminants, and no useful molecular or deoxyribonucleic acid (DNA) markers exist for the determination or the detection of the effects of chromium VI on soil-dwelling nematodes.

Hexavalent chromium (chromium VI) exists in soils naturally and is the sixth most abundant element in the Earth's crust. It is also present as a result of human practices, and mostly from those practices associated with industry. Chromium occurs naturally in Irish agricultural soils in concentrations between 5 and 250 ppm (McGrath et al., 2001), and in various soil types ranging from 1.04 to 3,015 ppm worldwide (Coleman, 1988; Richard and Bourg, 1991). Hexavalent chromium has been extensively shown to induce general environmental toxicity, as well as more specific effects of an acute and chronic nature such as neurotoxicity, dermatoxicity, genotoxicity, carcinogeneticity, and immunotoxicity (von Burg and Liu, 1993; Barceloux, 1999). It is believed that chromium inflicts most damage during reduction of chromium VI to chromium III, a process considered to be initiated in the cell by glutathione (Bose et al., 1992; Stearns and Wetterhahn, 1994; Moghaddas et al., 1995). Studies have suggested that hexavalent chromium contamination adversely affects the lifespan of fish (Perez-Benito, 2006), can initiate behavioural and biological changes in earthworms (Sivakumar and Subbhuraam, 2005), and cause serious morphological and biochemical alterations in algae (Devars et al., 1998; Rai and Rai, 1998; Okamoto et al., 2001).

Important genetic *loci* for normal cellular function, such as the β-tubulin genes, may be potential candidates for the development of ecotoxicological molecular markers, and thus deserve further investigation. The β-tubulin genes are involved in many crucial cellular

processes and in nematodes there can be as many as five different functional specific isotypes in existence (Gogonea et al., 1999). Polymerase chain reaction (PCR) primers for the amplification of these genes from *S. feltiae* were developed by Boyle (2007) and, as a result, different fragments can be accessed to assess the levels of DNA change or damage inferred by toxicants such as chromium.

Although the β-tubulin genes may be adversely affected by chromium, a more powerful approach is to isolate genes that are specifically expressed by the nematode in the presence of chromium VI. This can be performed by generating a subtracted cDNA library enriched for such genes. This material can then be employed to help understand the molecular consequences to the organism when exposed to chromium VI. Furthermore, these genes when isolated can be developed further as chromium VI-specific molecular markers that can be employed, in conjunction with the fitness parameters described above, to assess a contamination event in Irish soils in the context of the consequences to this nematode, which can be representative of consequences to other levels of the biosphere.

1.2 Project Aims

The overall aims of this study were:

- To investigate the effect of chromium VI on developing populations of *S. feltiae* in vivo within infected host cadavers.
- To investigate the effects of chromium VI on the IJ of *S. feltiae* as it exists in contaminated soils. The traits to be investigated were reproductive potential, development times, infectivity and survival rates within contaminated soils in order that they could be evaluated as suitable sublethal toxicity end points.
- To develop a library of genes specifically expressed in the presence of chromium VI in order to understand the effects of chromium VI at the molecular level within the organism.
- To establish the gene library as a valuable national and international resource for the development of molecular tools as biomarkers and for the initiation of subsequent investigations on specific genes and chromium VI toxicity.

2 The Effects of Chromium VI on the Nematode *Steinernema feltiae* during In Vivo Development within Host Cadavers, and on the β-Tubulin Genes

2.1 Introduction

Steinernema feltiae spends the entire developmental period of its life cycle in vivo within a suitable host cadaver. It is here that the nematode feeds and reproduces to produce large numbers of progeny which then emerge en masse from the cadaver. The nematode will infect, kill and feed in largely below-ground larval insects. These larval insects may be exposed to any soil contaminants present, and would therefore present a contaminated food source for the nematode. The infective stage of the nematode, with its physiological adaptations to withstand adverse environmental conditions, may not indeed be initially affected by any contaminants present. With the loss of its cuticle post host infection, and when feeding and reproduction has resumed, presumably the nematode becomes more susceptible to adverse contaminants that may have bioaccumulated within the infected host.

In light of this, the effects of varying concentrations of chromium VI on the developing and feeding nematode in vivo within host cadavers were investigated.

It was demonstrated that *S. feltiae* exhibits a high degree of resistance to chromium VI in vivo. Suitable sublethal end points were found to be the nematode reproductive potential and the rate of nematode development in the presence of the toxicant. An investigation into the occurrence of increased DNA variations between populations of a strain of *S. feltiae* cultured in the presence of chromium was also conducted.

2.2 Materials and Methods

2.2.1 *Culturing of nematodes*

An Irish isolate of *S. feltiae* (Strain 12(1)) (Boyle, 2007) was cultured at room temperature in *G. mellonella* and the resulting IJs were collected and subsequently washed with double-distilled deionised water. Infective juveniles were allowed to settle to the bottom of 50-ml sterile disposable centrifuge tubes (Sarstedt) and water was aspirated using a Pasteur pipette. Clean water was then added to the nematodes. This procedure was repeated at least three times, or until clear water was achieved after the IJs settled to the bottom of the tubes.

2.2.2 *Exposure of IJs to chromium VI in water*

Experiments were carried out in an effort to determine the levels of toxicity of chromium VI to the non-feeding IJ stage.

Concentrations of chromium VI were prepared in water from sodium dichromate (Lennox) ranging from 200 to 3,000 ppm in increments of 200 ppm. One millilitre of each was placed into a separate well of two 24-well plates. A nematode suspension was prepared containing approximately 300 nematodes in 10 μl, and 10 μl of this suspension were added to each treatment well of the 24-well plates. Exposure experiments were replicated three times, comprising a total of 48 treatments. Controls comprised *S. feltiae* IJs incubated with treatments but in water only. All pipette tips were flushed with 2% Triton X-100 directly before use so as to minimise nematodes adhering to the inside of the tip. All treatments were incubated at room temperature (20–22°C) for 24 h in the absence of direct light.

After incubation, each treatment replicate was mixed by gently flushing the nematode suspension in and out of a pipette tip; 20 μl were then taken and live and dead nematodes were counted visually using a light stereoscope. Six counts were performed per treatment in this manner and means were calculated.

2.2.3 *Exposure of nematodes to chromium VI during in vivo development*

Last instar larvae of *G. mellonella* were weighed to ensure that all larvae were in the 250–350 mg weight range. Each was injected with approximately 10–15 IJs of *S. feltiae* Strain 12(1). After larval deaths, which

occurred approximately 24 h after the injections, the cadavers were weighed again and the weights were recorded. Sodium dichromate was then injected into larvae to give concentrations of chromium VI from 200 to 3,000 ppm per cadaver weights at increments of 200 ppm. Treatments were replicated five times. Controls were also prepared where *S. feltiae* Strain 12(1) was injected into larvae but with no subsequent chromium injections, and a separate set of controls was prepared where larvae were injected with only 10 µl of sterile double-distilled deionised water. All controls consisted of five replicates of each.

All wax moth larvae cadavers were placed individually onto separate White traps (White, 1927), labelled with the associated recorded weights, and stored at room temperature (20–22°C) in the absence of direct light.

2.2.4 IJ emergence times

The time taken for IJs to emerge was assessed by recording the time, in days, at which IJs were first observed in the water reservoirs at the bottom of the White traps. This involved visually recording with a light stereoscope the nematodes present in the water at a consistent and single observation time each day. In this case, the observations were carried out once at 11:00 h each day of the trials.

2.2.5 Total IJ numbers (reproductive potential)

The following procedure is taken from that described by Rolston (2004). Briefly, 7 days after emergence was first observed, the contents of the White traps were decanted into 50-ml centrifuge tubes (Sarstedt) and washed as previously described. After three washes (or more if needed), centrifuge tubes with nematodes were filled to a total volume of 50 ml with sterile double-distilled and deionised water. This nematode suspension was homogenised by inversions and six aliquots of 10-µl volumes were taken and pipetted onto a clean Petri dish. Nematodes present in each aliquot were counted visually using a light stereoscope and results recorded. The approximate total count of IJs present in the White trap reservoirs was calculated and, using the weights recorded at the point of sodium dichromate injections, the approximate nematode count per milligram of cadaver (host insect) was calculated.

2.2.6 Extraction of DNA

Nematodes were collected from the reproductive potential trials for DNA extraction. The quantity of nematodes available varied, but there were generally adequate amounts collected for extraction. Those that yielded lesser amounts, such as the 1,600-ppm treatment, produced enough nematodes after extended emergence and harvesting. Generally, approximately 50–150 mg of a fresh or frozen nematode pellet were crushed in a sterile mortar with a pestle. Following this, 1.5 ml of lysis buffer (0.1 M Tris–HCl pH 8.5, 0.05 M EDTA, 0.2 M NaCl, 1% SDS, 100 µg/ml Proteinase K) were added and mixed with the nematode paste by gentle swirling. Using a pipette, this mixture was removed to a clean, sterile 50-ml polypropylene centrifuge tube and incubated overnight at 37°C. An equal volume of buffer-saturated phenol was added and the solution was extracted for 10 min by gentle rolling back and forth on a bench. The solution was centrifuged for 15 min at 10,000 × *g* and the upper aqueous phase carefully removed and placed into a new clean, sterile 50-ml polypropylene centrifuge tube. An equal volume of phenol/chloroform (1:1) was added to the aqueous phase and extracted and centrifuged as before. This time the upper aqueous phase was removed and placed into sterile microcentrifuge tubes. An equal volume of chloroform/isoamyl alcohol was added to the aqueous phase and solutions were extracted and centrifuged as before, only this time with a microcentrifuge. The upper aqueous phase was removed, placed into a fresh microcentrifuge tube and DNA was precipitated from solution by the addition of 2 M sodium acetate (to a final concentration of 0.2 M) and two volumes of 95% molecular biology grade ethanol pre-chilled to –20°C. Solutions were mixed thoroughly and then incubated at –20°C overnight. The sample was then centrifuged for 15 min at 14,000 × *g* and the supernatant discarded. The resulting DNA pellet was washed with 70% ethanol followed by air-drying in a DNA-clean hood for up to 1 h. The pellet was re-suspended in 300 µl TE buffer (Tris–EDTA pH 8: 10 Mm Tris–HCl, 1 Mm EDTA). The re-suspended pellet was treated with an RNase solution (2 mg/ml) in a 1:100 (v/v) ratio. This was incubated at room temperature for 1 h. Following incubation, the solution was extracted with phenol/chloroform and chloroform/isoamyl alcohol as

previously described, and sodium acetate/ethanol precipitations were performed also as described previously. The final ethanol-washed DNA pellet was air-dried at room temperature for 2 h and re-suspended in sterile double-distilled and deionised water and, either used immediately, or stored at either 4°C or –20°C for future use.

2.2.7 PCR amplification of the β-tubulin genes

Two primers which were designed for the amplification of β-tubulin genes from *S. feltiae* (Boyle, 2007) were used for this study. The sequences of these primers are as follows: Tub1AfwdSpc: GCG GTA ATC AGA TCG GTT C; Tub4ArevSpc: GCG GTC GGG GTA TTC TTC GCG. The PCR conditions were: 5 mM final concentration of $MgCl_2$, 0.2 mM final concentration of dNTP (0.05 mM each of dTTP, dGTP, dCTP, dATP), 50 pmol of each primer, 50–100 ng template DNA, 1.25 units of *Taq* DNA polymerase (GoTaq Polymerase, Promega), a 1× final concentration of associated PCR buffer, and sterile double-distilled water to a final volume of 50 μl. The thermocycling conditions were as follows: an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 57°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 5 min. The results were visualised by electrophoresis using 1.5% agarose stained with 0.5 μg/ml ethidium bromide.

Samples to be sequenced were processed and sent according to specific contract sequencer requirements (Qiagen, Germany) and the results were returned as a compressed file via e-mail.

2.2.8 Statistical analyses

Statistical analyses were conducted using SPSS (Version 12.0.1 for Windows) and Microsoft Excel XP Professional 2002 (Version 5.1 for Windows). Specifically, tests of significant fit of data to the normal distribution were determined by the Kolmogorov–Smirnov test (SPSS). The significance of data from IJ exposure to chromium VI experiments, and the emerging IJ numbers experiment, was tested using the Kruskal–Wallis test for non-parametric data (SPSS). Pairwise analysis was performed on data from the emerging IJ numbers experiment using the Mann–Whitney *U* test (SPSS). Correlations between time of emergence and chromium VI concentrations, and between emerging numbers and chromium VI concentrations were tested using the Kendall's Tau-*b* test for non-parametric data (SPSS). Means, standard deviations, standard errors and charts were calculated using Microsoft Excel.

For the sequence analysis experiments, DNA sequences were analysed using ClustalW (Pearson and Lipman, 1988) and MEGA, Version 3.1. Tests to investigate the null hypothesis of the neutrality of observed DNA mutations were performed using Tajima's neutrality test (Tajima, 1989).

2.3 Results

2.3.1 Fitness trials

There were significant differences in nematode survival between treatments where IJs were exposed to different concentrations of chromium VI (Fig. 2.1) (Kruskal–Wallis test: $P < 0.01$ confidence interval, $H = 43.419$, $df = 15$). However, although IJ deaths were observed in the presence of chromium VI from concentrations of 400 ppm, survival rates did not appear to be radically reduced until at the relatively high concentration of 1,000 ppm and higher. At 1,600 ppm, survival rates dropped to below 20% and remained so with increasing chromium VI concentrations. Interestingly, above 1,600 ppm, there was a slight increase in survival numbers. This was not maintained after 2,400 ppm chromium VI.

When *S. feltiae* was exposed to varying levels of chromium VI in vivo (during the life cycle inside the host insect), results once again indicated a high level of resistance (Fig. 2.2) (Kruskal–Wallis test: $P < 0.01$ confidence interval, $H = 55.60$, $df = 15$). Nematodes appeared to develop and reproduce in numbers comparable to the controls up to concentrations of 1,000 ppm (Mann–Whitney *U* test: $P > 0.05$). There was an increase in numbers of IJs emerging compared with the controls in concentrations up to 600 ppm, and a reduction in emerging numbers compared with the controls at 800 ppm and higher. Neither of these variations was statistically significant, however (Mann–Whitney *U* test: $P > 0.05$). Pairwise analysis of the data revealed that concentrations of 1,600 ppm chromium VI and above resulted in a reduction in IJ numbers, compared with the controls, that was statistically

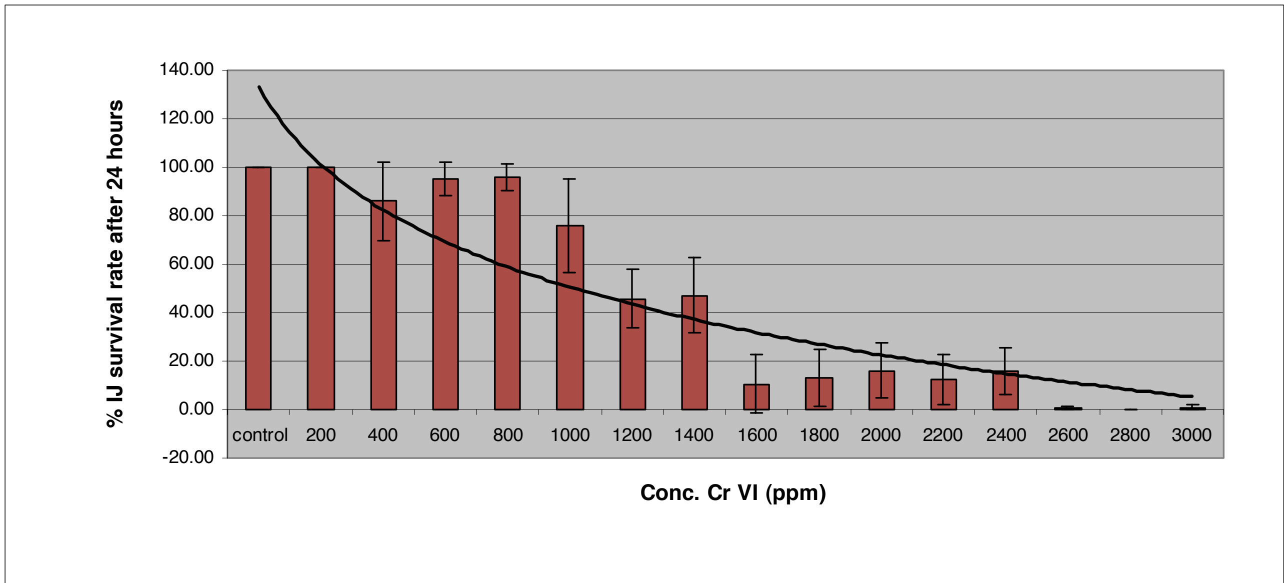

Figure 2.1. Lethal concentration at 50% (LC_{50}) test results on *Steinernema feltiae* infective juveniles (IJs) in the presence of varying chromium VI (Cr VI) concentrations in water over a 24-h period.

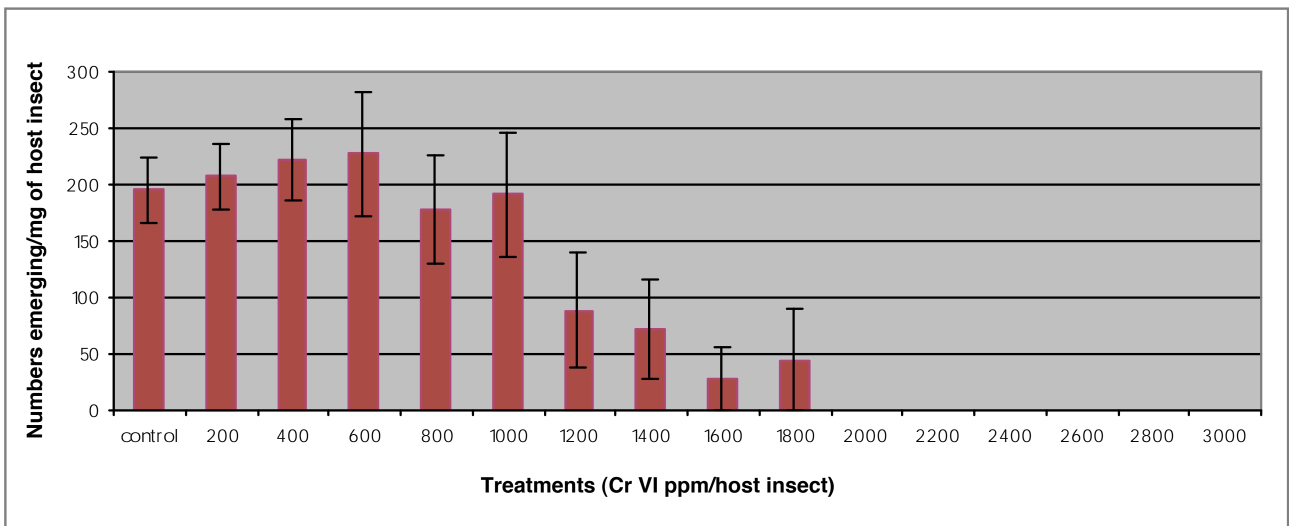

Figure 2.2. Mean of emerging numbers of *Steinernema feltiae* infective juveniles from host insects (*Galleria mellonella*) contaminated with increasing concentrations of chromium VI (Cr VI).

significant (Mann–Whitney *U* test: $P < 0.05$). At concentrations of 2,000 ppm and higher, there was no nematode emergence. In cases where no emergence was observed, there was substantial eventual colonisation of the host cadavers by other micro-organisms that resulted in the eventual degradation of the cadaver.

A clear increase in nematode emergence times was observed with increasing chromium VI concentrations (Fig. 2.3). There was a significant correlation between the times taken for IJs to emerge and the concentrations of chromium (Fig. 2.3) (Kendall's Tau-*b* correlation coefficient = –0.452, $P < 0.001$), and between numbers emerging and the concentrations of chromium (Fig. 2.2) (Kendall's Tau-*b* correlation coefficient = –0.599, $P < 0.001$).

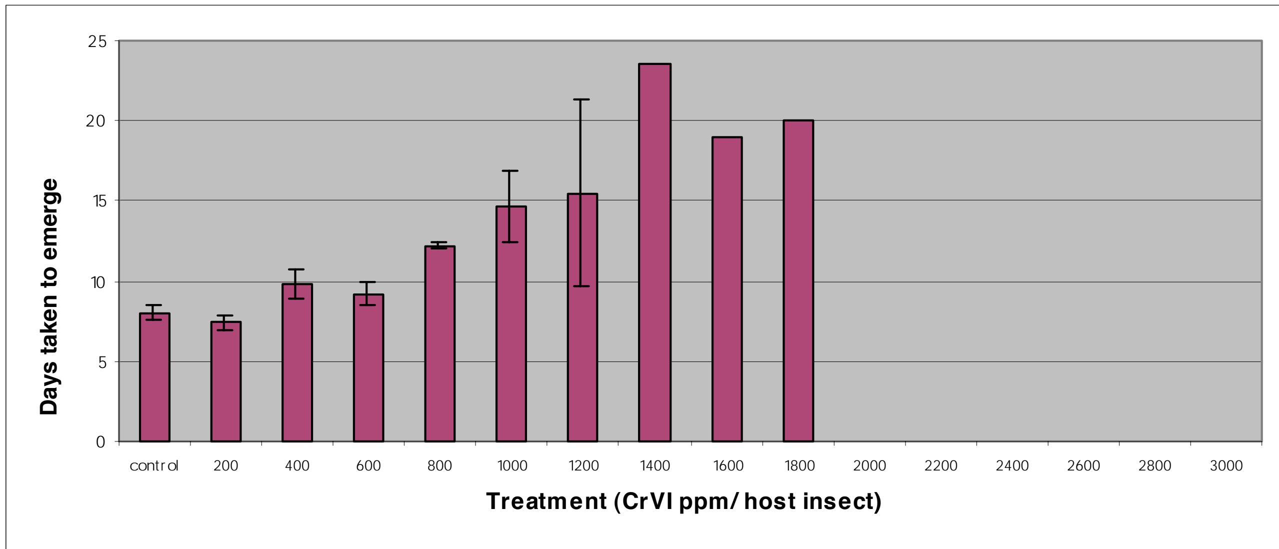

Figure 2.3. Mean number of days taken for *Steinernema feltiae* infective juveniles to emerge from host insects (*Galleria mellonella*) contaminated with increasing concentrations of chromium VI (Cr VI).

2.3.2 β-Tubulin gene analysis

Variations were observed among fragments of the β-tubulin genes which were amplified and sequenced from DNA isolated from emerging IJs from the described treatments (Fig. 2.4). Although the variations were not as many as one would have expected, they were sufficient to result in a rejection of the null hypothesis of neutrality (Tajima, 1989). The estimates of θ are 0.006477 per nucleotide based on S (the number of segregating sites) and 0.0026 based on π (the average pairwise difference). The estimates are significantly different from one another, and therefore are not consistent with neutrality (Tajima's D statistic of -1.7113, $P < 0.01$).

2.3 Discussion

From the results presented in this report, it is apparent that the EPN *S. feltiae* exhibits considerable resistance to hexavalent chromium. A previous study reported that metal ions such as lead, cadmium, zinc and copper had observable negative effects on *S. feltiae* IJs when they were exposed to them in water for 96 h (Jaworska and Gorczyca, 2002). The authors also reported that manganese had a positive effect on the IJs resulting in improved infectivity and reproduction potential after exposure. In the experiments reported from this study there was also a slight increase in nematode reproduction potential compared with the controls between 200 and 600 ppm (Fig. 2.2). This finding is interesting in that the supposed contaminant appears to actually aid the organism at certain concentrations although, because statistical significance was not observed, this statement must be made with caution.

Members of the order Rhabditida, of which *S. feltiae* is a member, are believed to display a generally high level of resistance to toxicants due to the close symbiotic relationships with bacteria that result in regular contact with potentially toxic end products (Bongers and Ferris, 1999). In addition, the IJs of steinernematid species possess a thick cuticle and a closed alimentary canal. Such characteristics are believed to confer resistance to extreme environmental conditions and to prevent the entry of pathogens and reduce water loss during their existence in soils (Poinar, 1990). *Steinernema feltiae* IJs are also not affected by osmotic stresses created while in water, unlike *S. feltiae* adults (personal observations). It is quite possible that these adaptations also serve to protect the IJs from toxicants such as chromium VI. However, it would be prudent to note that there were also high chromium VI resistance levels detected for *S. feltiae* during the in vivo exposure trials (Fig. 2.2). This may indicate that although the specific adaptations of an *S. feltiae* IJ confer protective advantages while in the soil, there may in fact be other

200(1)	GCG	GTA	ATC	AGA	TCG	GTT	CTA	AGG	TGA	GGC	TCT	CTG	CTT	GCG	TAG	45
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
1200(2)	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	45
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45

200(1)	TAC	CTG	TTC	AGT	TTC	TAT	CGT	TAT	CCT	TAT	AGT	TCT	GGG	AGG	TCA	90
200(2)	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	90
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
1000(4)	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	..G	90
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	90

200(1)	TCT	CAG	ACG	AAC	ACG	GAA	TCG	ACC	CTA	CCG	GAA	CGT	ATA	ACG	GCG	135
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	135

Figure 2.4. Deoxyribonucleic acid (DNA) sequence alignment of β-tubulin gene fragments. Fragments were polymerase chain reaction amplified from DNA isolated from *Steinernema feltiae* populations that were cultured in the presence of varying concentrations of chromium VI. Sequence titles reflect concentrations of chromium VI present in source samples, and numbers in brackets represent replicate numbers. Red type highlights variations.

200(1)	ACA	GCG	ATC	TGC	AAC	TCG	AGC	GCA	TCA	GCG	TTT	ACT	ACA	ATG	AAG	180
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
600(4)	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	180
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
1000(1)	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	180
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
1200(5)	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	180
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180
1600(1)	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	180
Control	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	180
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	180

200(1)	CCG	CTG	CCG	GAA	AGT	ACG	TCC	CAC	GTG	CCG	TCC	TGG	TCG	ACT	TGG	225
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
400(5)	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	225
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
1400(2)	...	...	...	...	...	...	...	...	...	...	...	..-	...	...	...	225
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225
Control	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	225
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	225

200(1)	AGC	CCG	GAA	CGA	TGG	ACT	CGG	TTC	GCG	CCG	GGC	CCT	ATG	GAC	AAC	270
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	270

Figure 2.4 *contd.*

200(1)	TTT	TCC	GGC	CAG	ATA	ATT	ACA	TCT	TTG	GGC	AAA	GTG	GCG	CTG	GCA	315
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
1000(1)	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
Control	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	315
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	315
200(1)	ACA	ATT	GGG	CGA	AGG	GTC	ATT	ACA	CGG	AGG	GAG	CTG	AAT	TGG	TCG	360
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
600(4)	...	...	...	...	...	...	...	...	...	...	...	.C.	...	...	...	360
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	G..	...	...	360
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	360
200(1)	ACA	ATG	TCC	TCG	ACA	TCG	TGC	GCA	AAG	AGG	CTG	AAT	CAT	GTG	ACT	405
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
1000(4)	...	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	405
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	405

Figure 2.4 *contd.*

200(1)	GCC	TTC	AGG	TGA	GCA	CCG	TGT	GGG	GAC	TAC	GGT	AGT	TAC	AGT	ATG	450
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	G..	...	450
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	450

200(1)	CCC	TCA	GGG	TTT	CCA	AAT	GAC	TCA	CTC	GCT	CGG	AGG	AGG	CAC	GGG	495
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	495

200(1)	ATC	CGG	AAT	GGG	AAC	TTT	GCT	GAT	CTC	AAA	AAT	CCG	CGA	AGA	ATA	540
200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
400(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
400(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
600(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
800(3)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
800(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
1000(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
1000(4)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
1200(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
1200(5)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
1400(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
1400(2)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
1600(1)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
Control	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540
Control2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	540

Figure 2.4 *contd.*

200(1)	CCC	CGA	CCG	C	550
200(2)	...	...	...	.	550
400(3)	...	...	...	.	550
400(5)	...	-..	...	.	550
600(1)	...	...	...	.	550
600(4)	...	...	...	.	550
800(3)	...	...	...	.	550
800(4)	...	...	...	.	550
1000(1)	...	...	...	.	550
1000(4)	...	...	...	.	550
1200(2)	...	...	...	.	550
1200(5)	...	...	...	.	550
1400(1)	...	...	...	.	550
1400(2)	...	...	...	.	550
1600(1)	...	...	...	.	550
Control	...	...	...	.	550
Control2	...	...	...	.	550

Figure 2.4 *contd.*

factors at work that allow survival during and after heavy metal exposure while in development within a suitable host insect.

Once an IJ invades a targeted host insect, it moults to a fourth-stage juvenile and begins feeding. Such developments presumably increase the vulnerability of the nematode to a range of deleterious factors as a result of the loss of the impermeable cuticle and a resumption of food intake. Yet, despite this, *S. feltiae* can still produce offspring relatively normally in the presence of 1,000 ppm chromium VI. In these experiments IJ production was observed even at concentrations as high as 1,800 ppm, although reduced reproductive potential was observed at these higher concentrations. Concentrations of 200 ppm or less were observed as being highly toxic to the host insect *G. mellonella* (personal observations). This fact necessitated a particular technique in order to introduce chromium VI to developing nematodes; chromium could only be injected into the host after death occurred due to IJ infection. In cases where chromium was injected first, almost instant death of the insects occurred, creating a less than ideal environment for IJ colonisation. *Steinernema feltiae* performs best when host death occurs as a result of proliferation of the nematode symbiotic bacteria (Poinar and Thomas, 1966).

The effects of chromium on nematodes have so far not received much attention apart from work by Bakonyi et al. (2003) and Nagy et al. (2004). These studies showed a marked decrease in nematode density and taxon richness, a reduction in the maturity index (MI), as well as the structure index (SI), and the predator and omnivore (P + O) ratio in response to heavy metals. Measurements with these parameters strongly suggested that chromium was one of the more deleterious of the heavy metal contaminants and that its effects can persist over a number of years.

Williams and Dusenbery (1990) investigated the lethal concentration at 50% (LC_{50}) values of various heavy metals, including chromium, on *C. elegans* and reported a value of 156 ppm chromium at 24 h exposure. Their results suggest that chromium is among the heavy metals that are most deleterious to nematodes, as the authors reported that salts such as zinc, cadmium and arsenic have higher LC_{50} values than chromium. They also suggest in the context of the present study that *C. elegans* possesses a much lower resistance to chromium than does *S. feltiae*, as survival in the results presented here only begins to reduce considerably after approximately 1,200 ppm (Fig. 2.1). Therefore, if *in situ* nematodes were to be employed to obtain a more accurate indication of the effects of chromium in the environment and the soil biota, *C. elegans* would be most likely discounted as

an indicator as it cannot survive in concentrations that are often found naturally in Irish soils. Thus, the higher resistance of *S. feltiae* to chromium would allow for the use of natural field populations of this nematode to be employed as 'on-site' bioindicators, compared with *C. elegans* test nematodes which are largely used as 'off-site' organisms and in a laboratory setting.

The molecular effects of chromium have not been assessed for nematodes. In the experiments reported here, β-tubulin gene fragments from *S. feltiae* progeny emerging from the reproductive potential experiments were PCR amplified and sequenced. A number of variations were observed between the β-tubulin genes from the treatments although they were not as numerous as expected. These variations were observed to appear at different sites among the fragments in different sequencing experiments. Additionally, variations were inconsistent among replicates, further supporting the hypothesis that chromium will induce mutations in a random nature rather than affecting particular codons or base pairs. Consequently, these variations cannot be detected using site-specific techniques, e.g. restriction fragment length polymorphism (RFLP) analysis. Furthermore, the levels of variations did not appear to be consistent with increasing concentrations of chromium VI, ruling out the utilisation of the variations in a quantitative technique that could ascertain the levels of chromium VI contamination relative to DNA change.

The apparently slight effect of chromium VI on the β-tubulin gene structure in *S. feltiae* was surprising, given the importance and activity of these genes and their proteins in cell division, cellular communication and other crucial cell processes. However, although there appeared to be a low detection rate for variations within the β-tubulin genes of progeny that developed in the presence of chromium, this may not imply that these variations did not occur. Although there is evidence of sperm-specific β-tubulin genes (Rudolph et al., 1987), the functions (or isotypes) of the β-tubulin genes studied here are unknown. It is quite possible that the majority of mutations occurred in somatic cells of *S. feltiae* individuals during chromium exposure but not in germ-line cells. The mutations that were detected may well be a result of germ-line mutations that confer little disadvantage on the progeny. The delay in the development and emergence of progeny at higher chromium concentrations may be a result of a recovery period among the population, as selection removes those affected by damaging somatic cell DNA variations and allows progeny with increased tolerance to develop and eventually thrive. Indeed, population genetic theory shows that deleterious mutations will be removed from a population by natural selection (Clark et al., 1981; Cronin and Bickham, 1998). It would be informative to know in how many generations deleterious mutations would be removed from the genome of *S. feltiae*. Although this is currently not known, it would constitute a very interesting avenue for investigation to understand the molecular mechanisms of exposure to, and recovery from, chromium in this nematode.

Interestingly, a study of the mitochondrial DNA of the nematode *Pellioditis marina* from a heavy-metal-contaminated site also reported reduced population development with a reduced genetic diversity among the population (Derycke et al., 2007). The authors proposed a number of factors that may have resulted in such a limited genetic effect such as too low a toxicant concentration, the selective neutrality of the chosen markers and the limited duration of their experiments.

Long-term trials were not conducted in these experiments. It would, therefore, be a worthy study in the future to apply β-tubulin gene sequence analysis to nematodes isolated from long-term chromium-contaminated soils to investigate recurrent or increased germ-line cell DNA effects.

2.4 Conclusions

It is clear that developing populations of *S. feltiae* demonstrate considerable resistance to chromium VI in vivo. This was apparent from the development and production of large numbers of IJs in insect hosts prepared with concentrations of up 1,000 ppm chromium VI. However, the reduction in nematode emerging numbers and the increasing intervals of time of nematode emergence with increasing chromium VI concentrations do suggest that there is an effect. At concentrations between 1,000 and 1,800 ppm there was evidence of emergence but this ability was observed to reduce at each intervening interval.

Beyond a chromium VI concentration of 1,800 ppm, nematodes were not observed to survive initial infection.

The fact that this organism can survive exposure to high levels of such an environmentally relevant contaminant makes it a candidate species for chromium-related environmental protection activities. This may be achieved by:

(a) The establishment of sublethal end points, which take into account effects undetected with acute toxicity measurements; and

(b) The development of molecular markers for chromium detection.

To date, the β-tubulin genes show little predictable variations that may be used as molecular markers, although variations have been observed whose rate of occurrence is higher than the rate that may have been expected after intra-species and, indeed, intra-population analysis.

As *S. feltiae* appears to be far more resistant to chromium VI than *C. elegans*, it is possible that *S. feltiae* also exhibits higher chromium VI resistance levels than those exhibited by other nematodes. An organism that can survive in the presence of a toxicant holds good bioindication potential for toxicant effects if tangible molecular or physiological changes occur that can be detected and employed for quantitative and/or qualitative measurements.

Steinernema feltiae is commonly found in Ireland and can be easily isolated from the soil and cultured in the laboratory. A high level of chromium resistance, coupled with measurable biomarkers, genetic or otherwise, that demonstrate bioavailable chromium, would be a valuable tool for environmental risk assessment.

3 The Effects of Chromium VI on the Infective Juvenile Stage of *Steinernema feltiae* in Contaminated Soils

3.1 Introduction

Although *S. feltiae* spends the most productive sections of its life cycle within host cadavers, in another stage it spends a significant amount of time living outside the host cadavers. This stage, the IJ, lives in soils and sands, and is the stage that will most likely bear the brunt of exposure in any contamination event. The IJ is physiologically well equipped to deal with adverse environmental situations such as desiccation, UV exposure, actions of pathogens and predators, and temperature. The following experiments were conducted to help us understand the effects of chromium VI on the nematode as it exists in soils pre-host cadaver infection. The results describe the behaviour of the nematode in the presence of chromium VI in sandy soils in the laboratory.

3.2 Materials and Methods

3.2.1 Exposure of S. feltiae *IJs to chromium VI*

Steinernema feltiae (Strain 12(1), isolated from Bull Island, Ireland) was grown in suitable host larval insects (*G. mellonella* – LiveFoods Direct UK) and enough worms were eventually cultured to give a concentration of 2,000 nematode IJs per millilitre of water suspension.

Separately, a quantity (approx. 100 kg) of sandy soil was collected from Bull Island, Dublin, Ireland, and transported to the laboratory. All the sand was sterilised using an autoclave as follows. The sand was divided into bench-top autoclave bags in weights of 250 g each. A strip of autoclave tape was placed into the centre of the sand and the bags sealed. Bags were autoclaved in groups of five at a pressure of 1.5 kg force/cm^2 and a temperature of 120°C, which was maintained for 60 min. In cases where the autoclave tape originally placed into the sand indicated insufficient heat and pressure treatment, the autoclaving procedure was repeated with a lesser load. This sand was then placed into plant pots at a weight of 500 g per pot.

The sand in the pots was spiked with chromium VI (sodium dichromate) in aqueous solution to give final concentrations from 10 to 100 ppm in increments of 10. Controls were prepared also, consisting of sand and no chromium VI. Each treatment was replicated three times. The sand was then mixed with a further 10 ml of sterile water to obtain a mild damp consistency which was maintained throughout the experimental period. One millilitre of nematode suspension (approx. 2,000 worms in water) was added to each pot. The nematodes were allowed to equilibrate in this environment for 24 h at room temperature.

Three wax worms per pot were labelled using a permanent marker and placed into the respective plant pots. Pots were stored at room temperature (20–22°C) in the absence of direct light for the duration of the experiment (31 days). Each pot was sealed with modified plastic bench-top autoclave bags to minimise moisture loss.

The wax worms were observed for mortality each day. When a wax worm died it was removed and placed on a White trap (White, 1927), the date of death was recorded, and it was replaced with another appropriately labelled wax worm. This was continued for a period of 31 days. After wax worm cadavers were placed on White traps, they were observed every day for nematode emergence, characterised by the presence of living IJs on the cadaver exterior. Once nematode emergence was observed, a period of 7 days was allowed to pass before emerged nematodes were counted (see next section).

3.2.2 Reproductive potential

The following procedure is taken from that described by Rolston (2004). Briefly, 7 days after emergence was first observed, the contents of the White traps were decanted into 50-ml centrifuge tubes (Sarstedt) and washed by allowing nematodes to settle to a pellet and carefully decanting the remaining water. Fresh double-distilled deionised water was added. After three

washes in this manner (or more if needed) centrifuge tubes with nematodes were again filled to a total volume of 50 ml with sterile double-distilled and deionised water. This nematode suspension was homogenised by repeated inversions and six aliquots of 20-μl volumes were pipetted onto a clean Petri dish. Nematodes present in each aliquot were counted visually using a light stereoscope and results recorded. The approximate total count of IJs present in the White trap reservoirs was calculated.

3.2.3 IJ emergence/development times

Infective juvenile emergence was assessed by recording the first observation of live IJs on the exterior of the cadavers incubating on White traps at a single observation time each day. In this case, the observations were carried out once at 11:00 h each day of the trials. Development times were calculated as the difference between the time of observed original wax worm deaths in the plant pots and the time of emergence of IJs from cadavers on the White traps.

3.2.4 Infectivity

Infectivity was recorded as the amount of time in days taken for a wax worm to die after being placed into the contaminated sand with nematodes, and where the deaths were confirmed to be as a result of IJ infection as demonstrated by IJ emergence from cadavers on White traps.

3.2.5 Survival rates

Survival rates were calculated as the total time in days where nematode IJs were observed to be active on the wax worm hosts added to the plant pots (i.e. death of added wax worms that subsequently produced IJs when placed on White traps).

3.2.6 Data analysis

Statistical analyses were conducted using SPSS (Version 15.0 for Windows) and Microsoft Excel XP Professional 2007 (Version 5.1 for Windows). Specifically, tests of significant fit of data to the normal distribution were determined by the Kolmogorov–Smirnov test or the Shapiro–Wilks test, depending on sample size and analysis of kurtosis, skewness, and frequency distributions of the data (SPSS). The differences among the data groups were tested for significance using the Kruskal–Wallis test for non-parametric data and a one-way analysis of variance (ANOVA) where the data appeared to be parametric. Correspondingly, pairwise analysis was performed on data groups that were deemed to exhibit significant differences using the Mann–Whitney *U* test for non-parametric data, and using the Tukey's honestly significant difference (HSD) test when equal variances among the means were assumed, or the Games–Howell test when equal variances were not assumed, for parametric data. Statistical correlation tests were performed to determine relationships between data sets using the Kendall's Tau-*b* test for non-parametric data (SPSS), or Pearson's correlation for parametric data. Multiple regression analysis was performed using SPSS to attempt to predict which variables had the most influence on selected outcomes. Means, standard deviations, standard errors and all charts were generated using Microsoft Excel.

3.3 Results

3.3.1 Effects of chromium VI on reproduction of S. feltiae

There were significant differences between nematode progeny numbers when IJs from experiments were allowed to infect and reproduce in wax moth larvae (one-way ANOVA: $F(10,154) = 5.528$, $P < 0.01$) (Fig. 3.1). Interestingly, the increase in concentrations of chromium VI did not appear to have much effect on reproductive potential until a concentration of 70 ppm. However, although nematodes produced greater numbers from infected cadavers at higher concentrations of chromium VI, it was observed that the higher concentration of chromium VI also resulted in less wax worms killed as a result of nematode activity (Fig. 3.2). The number of wax worms killed that produced nematodes dropped from 50 larvae among the non-chromium-contaminated controls to a total of 16 larvae at 10 ppm and upwards.

Pairwise analysis revealed significant differences in nematode progeny numbers between the controls and between 70 and 90 ppm (Tukey's HSD: $P < 0.05$). At 100 ppm, nematode progeny numbers appeared to reduce. There was a significant positive correlation between increasing concentrations of chromium VI in soils and nematode progeny production (Kendall's Tau-*b* correlation coefficient = 0.327, $P < 0.001$) (Fig. 3.3) indicating that the presence of chromium VI

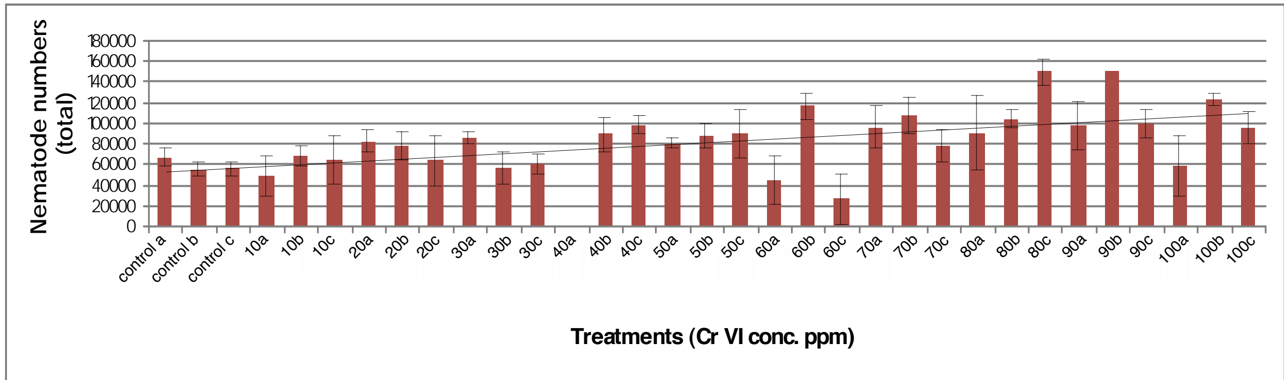

Figure 3.1. Data of mean values of nematode progeny numbers produced within host insects (*Galleria mellonella*) from *Steinernema feltiae* infective juveniles that were exposed to increasing concentrations of chromium VI (Cr VI). Standard errors shown. Denotations a, b and c signify experimental replicates.

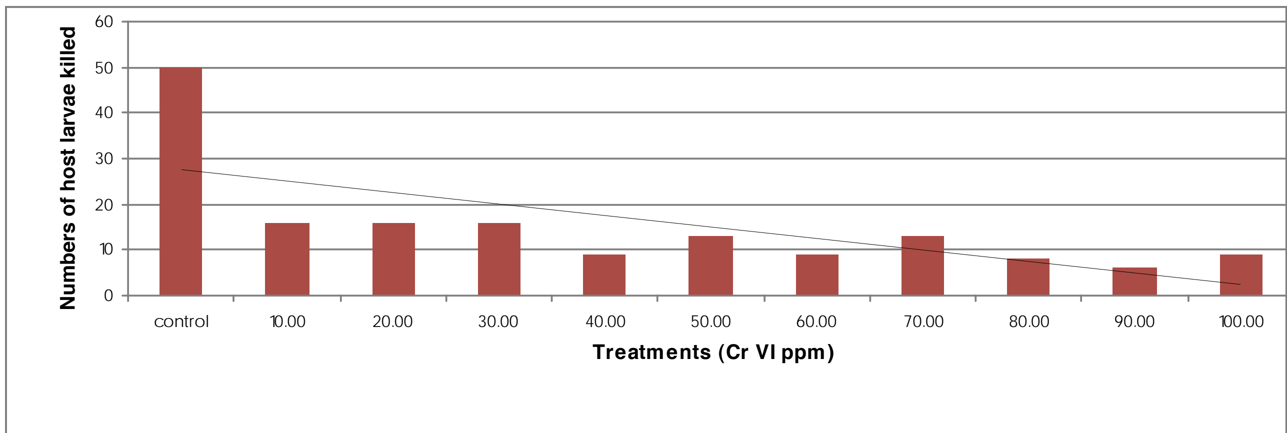

Figure 3.2. Total number of host insects (*Galleria mellonella*) killed by *Steinernema feltiae* infective juveniles that were exposed to increasing concentrations of chromium VI (Cr VI).

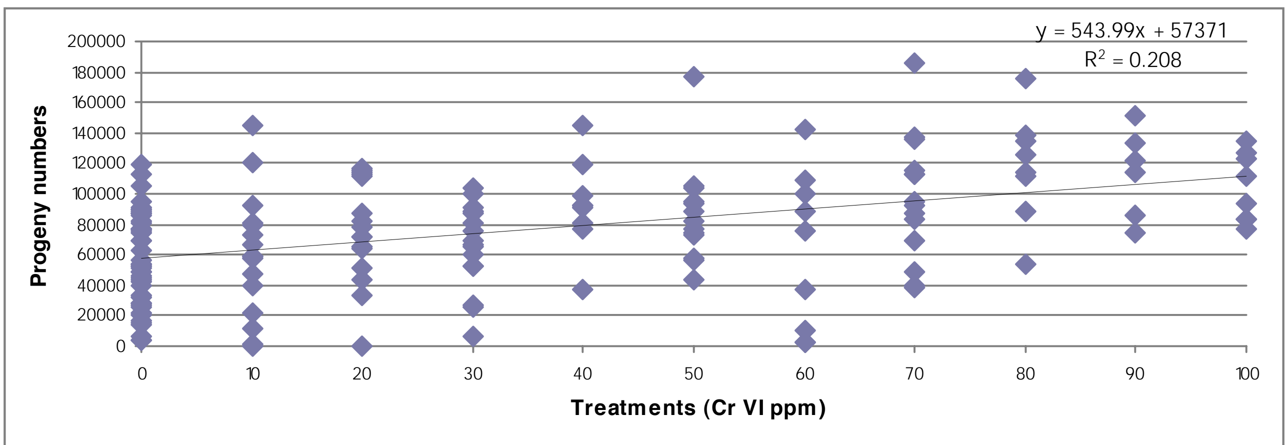

Figure 3.3. Scatter plot of bivariate correlation between increasing concentrations of chromium VI (Cr VI) and ultimate *Steinernema feltiae* progeny numbers (R^2 = 0.208) (Kendall's Tau-*b* correlation coefficient = 0.327, P < 0.001). Points on graph are of data generated from infective juvenile (IJ)-infected wax worms.

in an *S. feltiae* environment can have a positive influence on population numbers.

3.3.2 Effects of chromium VI on nematode development times

There were significant differences in the development times among the treatments (Kruskal–Wallis test: $P < 0.01$, $H = 34.363$, $df = 10$) (Fig. 3.4), suggesting that IJ exposure to increasing concentrations of chromium VI significantly slows down development of the nematode during reproduction. Pairwise analysis of the data appears to suggest that significant differences emerge between the controls and the treatments at 50 ppm upwards (Mann–Whitney *U* test: $P < 0.01$). However, this trend was not maintained at the 90-ppm treatment (Mann–Whitney *U* test: $P > 0.01$) yet was achieved once more at 100 ppm (Mann–Whitney *U* test: $P < 0.01$). Pairwise analysis did not reveal much significant difference among the mean development times between the differing concentrations of chromium VI.

There was also a significant positive correlation between increasing chromium VI concentrations and extended development times (Kendall's Tau-*b* correlation coefficient = 0.280, $P < 0.01$) (Fig. 3.5). Such a correlation proposes that nematode development times are significantly increased as a result of increasing chromium VI contamination.

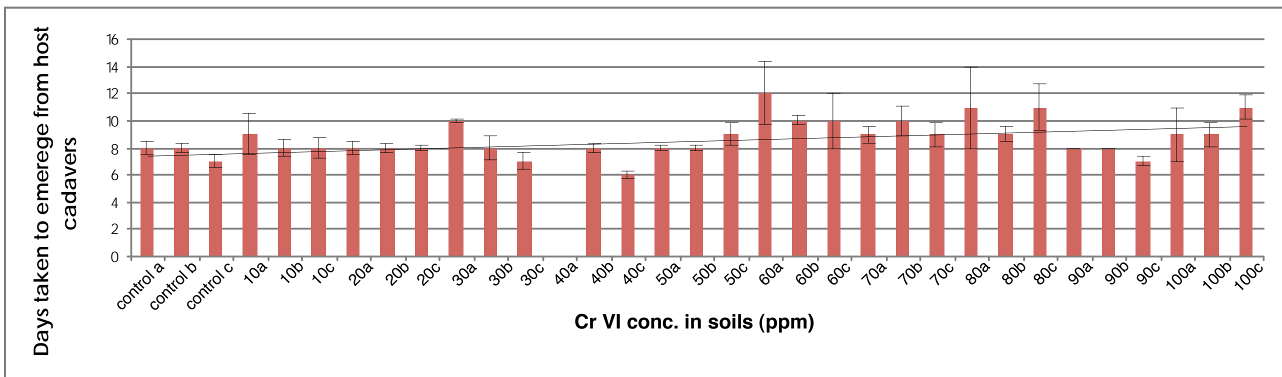

Figure 3.4. Data of mean number of days for *Steinernema feltiae* progeny to emerge (development times) from host larvae infected with infective juveniles that were exposed to increasing concentrations of chromium VI (Cr VI) in sand. Standard errors shown. Denotations a, b and c signify experimental replicates.

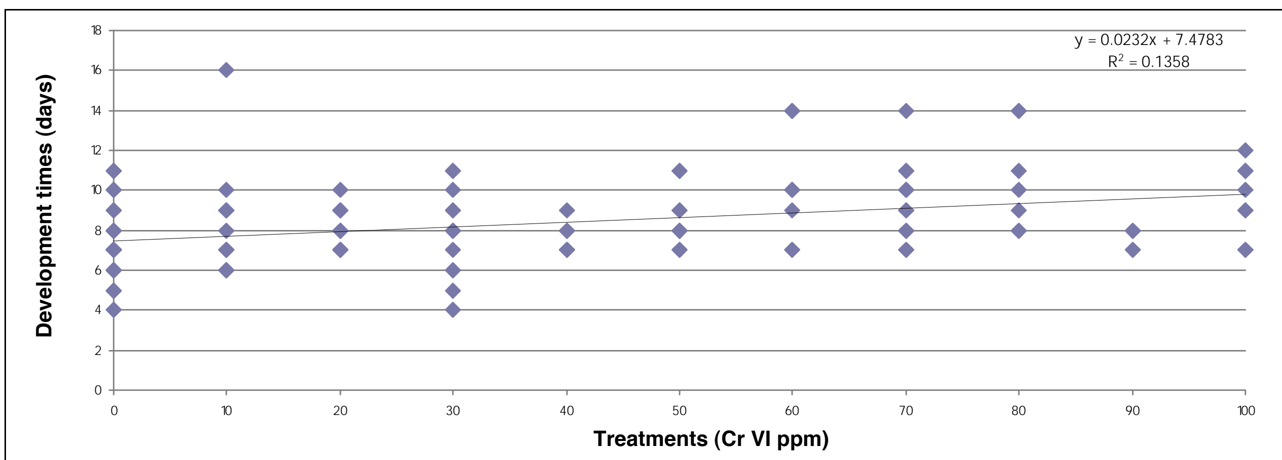

Figure 3.5. Scatter plot of bivariate correlation between increasing concentrations of chromium VI (Cr VI) and *Steinernema feltiae* development times ($R^2 = 0.1358$) (Kendall's Tau-*b* correlation coefficient = 0.280, $P < 0.01$).

3.3.3 Effects of chromium VI on infectivity

The infectivity of the nematode IJs on wax worms in chromium VI-contaminated soils is significantly reduced (Fig. 3.6) (Kruskal–Wallis test: $P < 0.01$, $H = 60.755$, $df = 10$). The particularly detrimental effects of chromium on infectivity are highlighted after pairwise analysis using the Mann–Whitney test where a concentration of 20 ppm upwards yields significant differences from the controls (Mann–Whitney *U* test: $P < 0.001$).

However, although not statistically significant, there are actually considerable reductions in infectivity rates from 10 ppm (Fig. 3.6). This can also be seen in the total wax worm numbers killed, another indicator of infectivity rates, over the entire experimental period (Fig. 3.2). Furthermore, correlation tests revealed a significant positive correlation between increasing concentrations of chromium VI and extended infectivity rates (Fig. 3.7) (Kendall's Tau-*b* correlation coefficient = 0.405, $P < 0.001$). Therefore, it appears that the ability of the

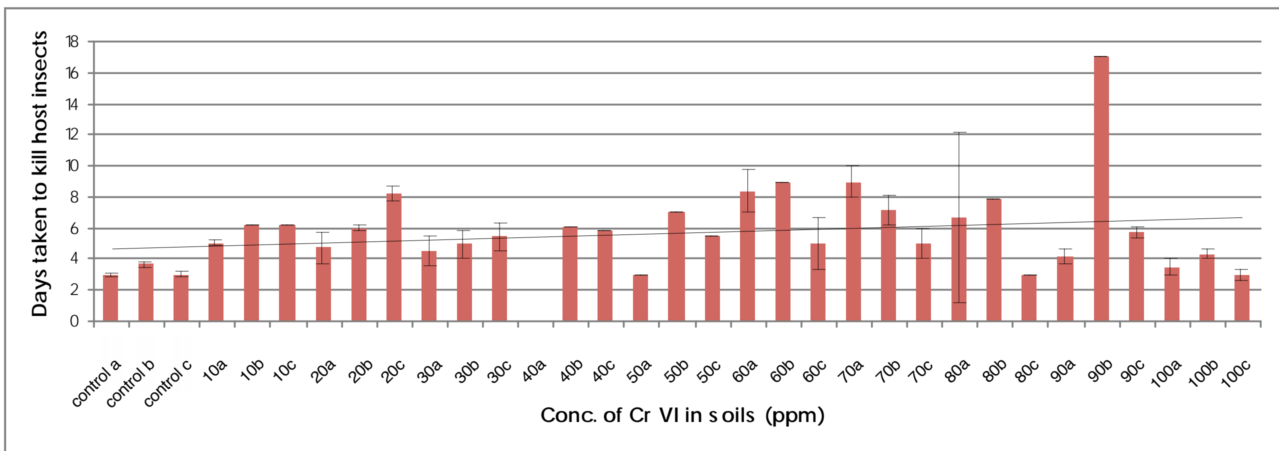

Figure 3.6. Data of mean number of days taken for *Steinernema feltiae* to kill host larvae (after addition into experimental plant pots) by infection with infective juveniles that were exposed to increasing concentrations of chromium VI (Cr VI) in sand. Standard errors shown. Denotations a, b and c signify experimental replicates.

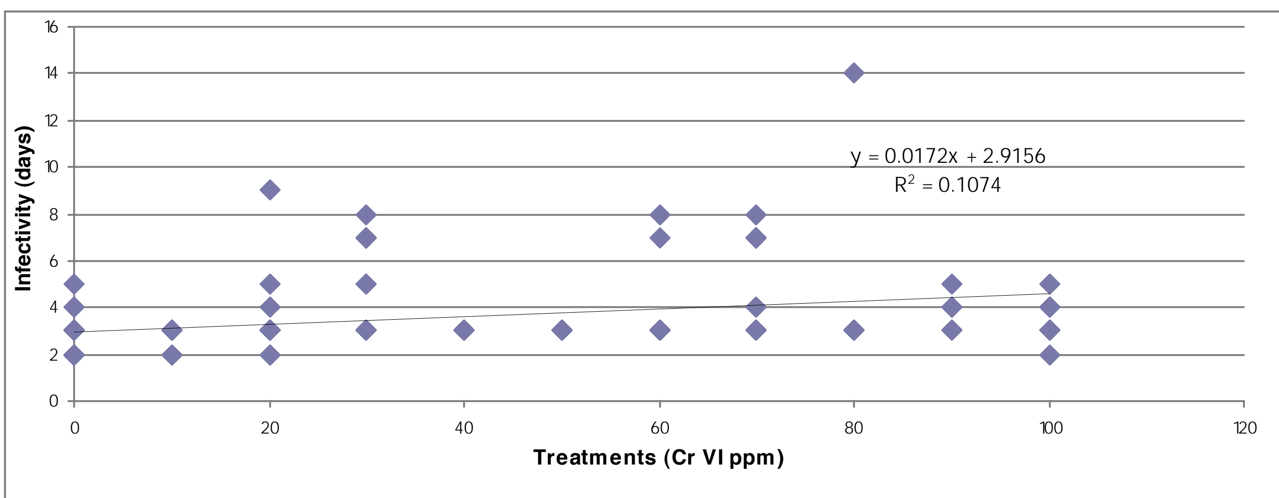

Figure 3.7. Scatter plot of bivariate correlation between increasing concentrations of chromium VI (Cr VI) and *Steinernema feltiae* infectivity rates ($R^2 = 0.107$) (Kendall's Tau-*b* correlation coefficient = 0.406, $P < 0.001$).

nematodes to remain active in the presence of chromium VI is severely curtailed, resulting in a reduction in the amount of potential cadavers within which to produce offspring.

3.3.4 Effects of chromium VI on survival times

Data for survival times exhibited a strong negative skew in the distribution and this interfered with statistical analysis. The interference was such that differences appeared not to be significant when tested with the Kruskal–Wallis test, despite a large difference between survival times and the controls (Fig. 3.8). Therefore, Mann–Whitney tests were performed on the data instead. Consequently, there were significant differences in survival times between the control samples and those of all the treatments (Mann–Whitney *U* test: $P < 0.05$). Among treatments, there were no significant differences in survival times observed (Mann–Whitney *U* test: $P > 0.05$).

There was a significant negative correlation between survival times and exposure of IJs to increasing concentrations of chromium VI (Kendall's Tau-*b* correlation coefficient = –0.410, $P < 0.001$) (Fig. 3.9). This negative correlation reflected the considerable reduction in the ability of the nematode IJs to withstand increasing concentrations of chromium VI over time as indicated by infection activity.

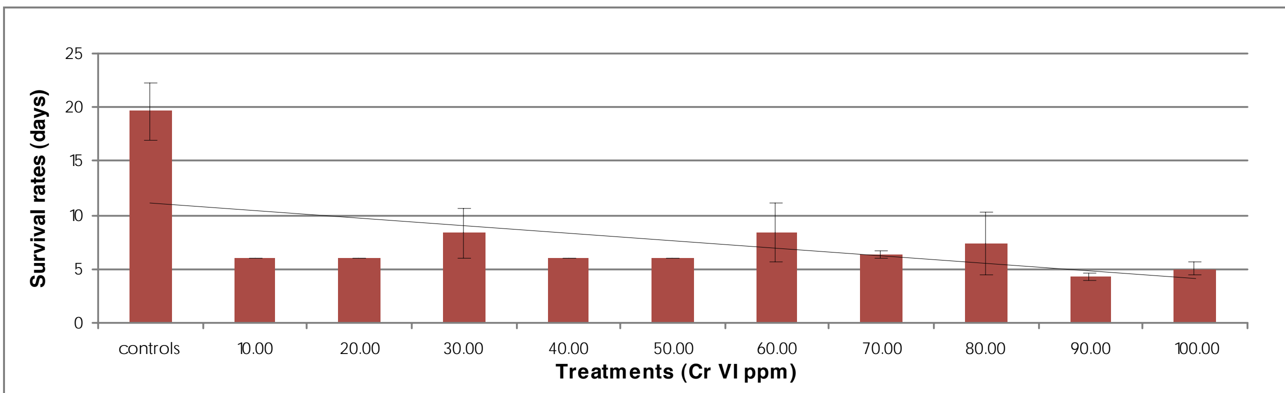

Figure 3.8. Data of mean survival rates of *Steinernema feltiae* infective juveniles (IJs) in increasing concentrations of chromium VI (Cr VI) as observed by infectivity. Data points are the time in days between the date nematode IJs were added to experimental plant pots and the date of last observed larval death due to IJ infection. Standard errors shown.

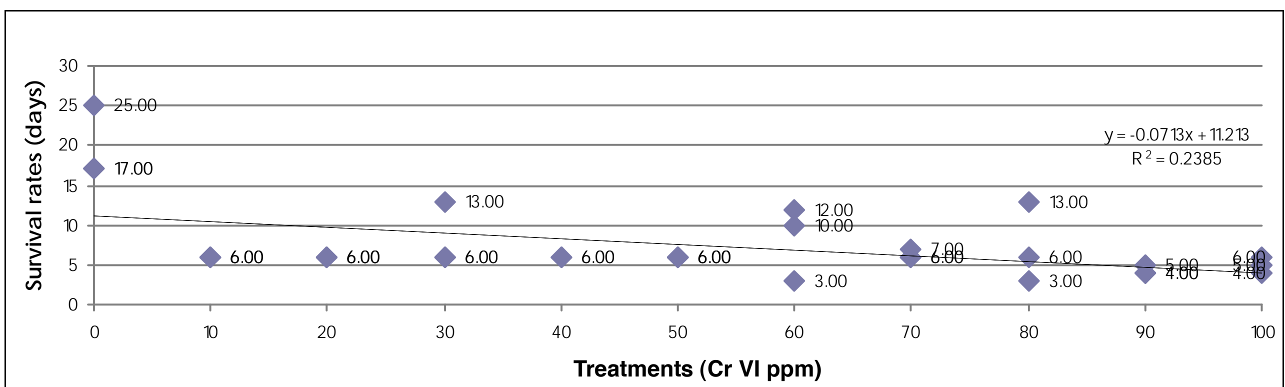

Figure 3.9. Scatter plot of bivariate correlation between increasing concentrations of chromium VI (Cr VI) and *Steinernema feltiae* survival rates ($R^2 = 0.2385$) (Kendall's Tau-*b* correlation coefficient = –0.410, $P < 0.001$).

3.3.5 Further correlations

Correlation tests were also performed on the data to determine if nematode progeny numbers were influenced by longer development times and if the longer development times corresponded with an increase in the rates of survival of IJs in chromium VI-contaminated environments.

Nematode numbers demonstrated a significant positive correlation with increasing development times (Kendall's Tau-*b* correlation coefficient = 0.160, P < 0.01) (Fig. 3.10). This correlation suggested a strong increase in numbers of IJs produced up to a period of 11 days development time.

However, when correlation coefficients between treatments and progeny numbers and between development times and progeny numbers are compared, it appears that the former has the stronger correlation and higher significance value (Kendall's Tau-*b* coefficient = 0.327, P < 0.001). This suggests that although nematode offspring can increase in numbers as a result of chromium VI exposure and also exhibit longer development times within a host cadaver, it appears that chromium VI may be the greater influencing factor in determining increasing nematode reproduction numbers.

To determine whether it is possible that longer nematode development times are a result of increasing chromium VI exposure times, a correlation test was performed between them. This test was performed using development times within treatment replication means. This was because survival times were recorded as the last day of observed IJ activity within the treatment pots and these figures could not be directly compared with all development time observations per corresponding treatments and replications due to a disparity in sample numbers between them. Therefore, correlations were made using mean IJ development times among all wax worms taken from each treatment replication *versus* final observed survival times for each replication treatment.

The results suggested that there was a slight decrease in development times as a result of increasing exposure of nematodes to chromium VI, although this was not statistically significant (Kendall's Tau-*b* correlation coefficient = –0.024, P > 0.05) (Fig. 3.11). From the scatter graph, it can be observed that the majority of treatments did not exhibit survival times extending beyond 5–6 days, and indeed the points at the extreme end of the plot corresponding to 20–25 days are those of the controls, where no chromium VI was included in the exposures.

It is clear that these points skew the data somewhat and the correlations were repeated after their removal. This time there was a positive correlation (Kendall's Tau-*b* correlation coefficient = 0.133, P > 0.05) (Fig. 3.12), although it was still not significant. These tests appeared to suggest that the longer the IJs are exposed to chromium VI, there is a slight increase in

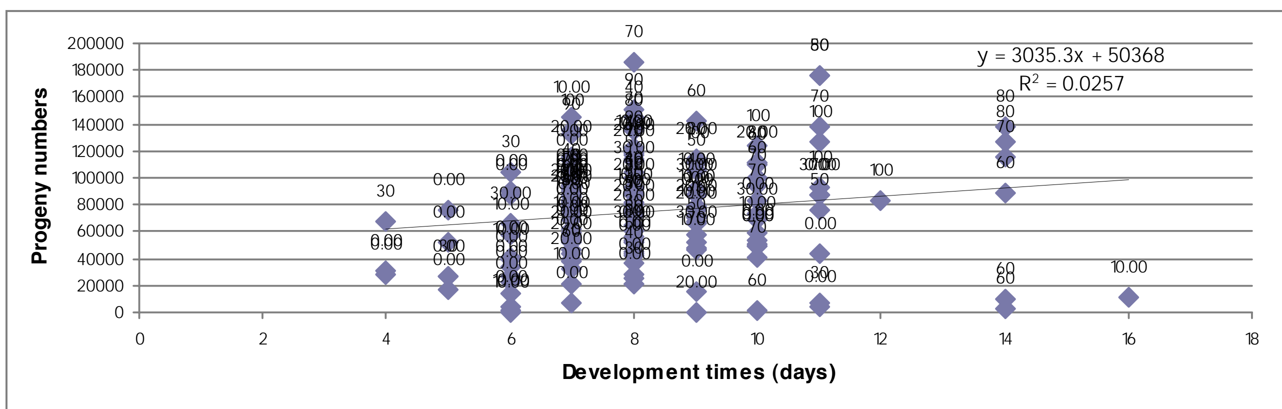

Figure 3.10. Scatter plot of bivariate correlation between nematode progeny development times (days) and corresponding *Steinernema feltiae* progeny numbers (R^2 = 0.0257) (Kendall's Tau-*b* correlation coefficient = 0.160, P < 0.01). Corresponding treatments are recorded at data points on graph.

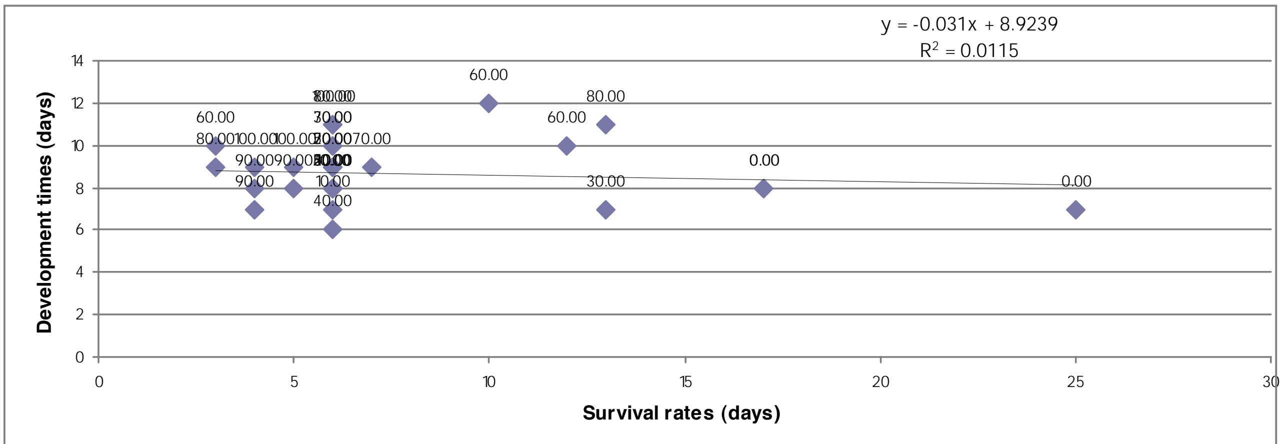

Figure 3.11. Scatter plot of bivariate correlation between nematode progeny development times (days) and corresponding *Steinernema feltiae* survival rates (days) (R^2 = –0.0115) (Kendall's Tau-*b* correlation coefficient = –0.024, P > 0.05). Corresponding treatments are recorded at data points on graph.

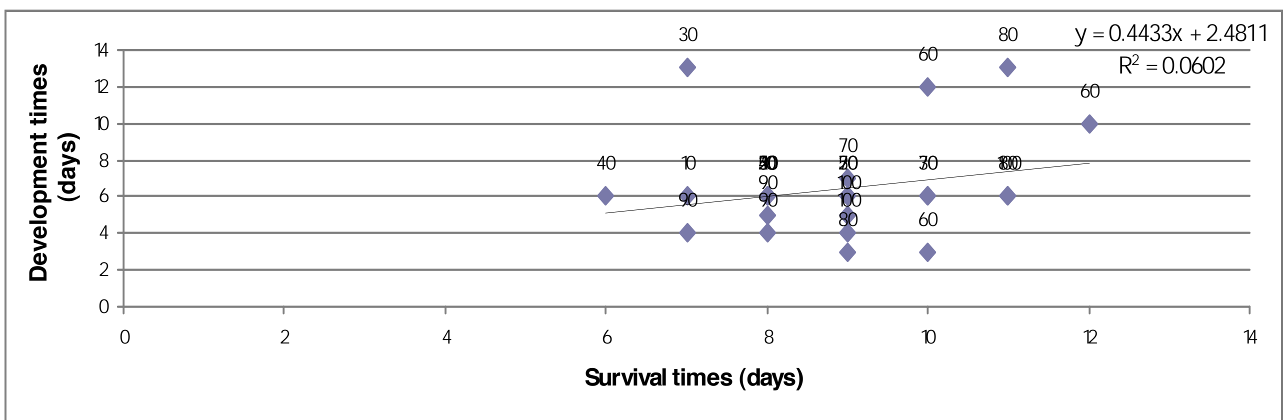

Figure 3.12. Scatter plot of bivariate correlation between nematode progeny development times (controls excluded) and corresponding *Steinernema feltiae* survival rates (controls excluded) (R^2 = 0.0602), (Kendall's Tau-*b* correlation coefficient = 0.133, P > 0.05). Corresponding treatments are recorded at data points on graph.

development times up to approximately 13 days. Indeed, it can be observed from the data that beyond 6 days there is a reduction in the number of wax worms killed by IJ infections. A correlation test on data from up to 6 days exposures yielded a negative correlation but which was also non-significant (Kendall's Tau-*b* correlation coefficient = –0.065, P > 0.05).

A correlation test was also performed to examine if there is any relationship between the survival rates and infectivity but the results were not statistically significant. A slight negative correlation was achieved, reflecting a drop in infectivity rates the longer IJs are exposed to chromium VI (Kendall's Tau-*b* correlation coefficient = –0.036, P > 0.05) (Fig. 3.13).

3.3.6 Multiple regression correlations

In order to predict which variable may be attributed as having the most influence on the nematode progeny numbers, multiple regression analysis was performed. Data were transformed by obtaining the square root and were used to compute the multiple regressions.

Multiple regression analysis revealed that only 15% (R^2 = 0.148) of the variance can be explained by the predictors, treatments (chromium VI concentration) and

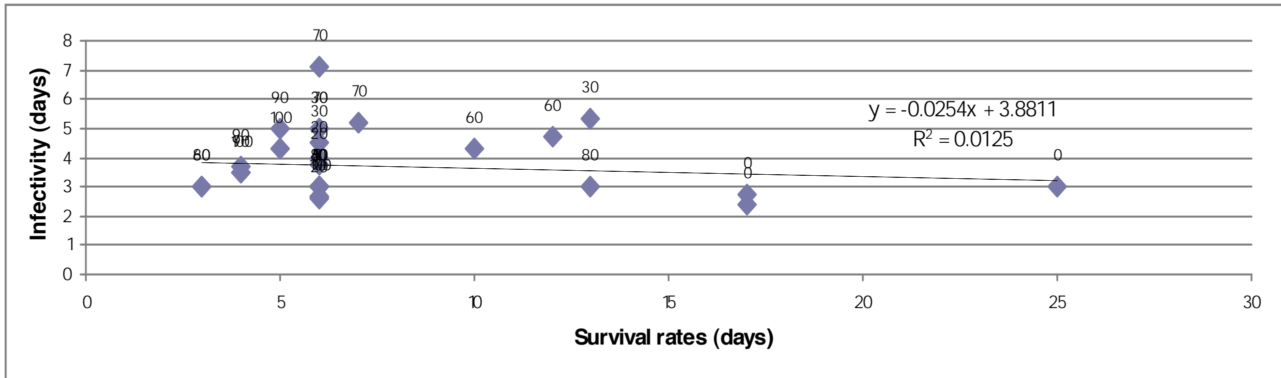

Figure 3.13. Scatter plot of bivariate correlation between nematode *Steinernema feltiae* survival rates and corresponding infectivity rates ($R^2 = 0.0125$) (Kendall's Tau-*b* correlation coefficient = –0.036, $P > 0.05$). Corresponding treatments are recorded at data points on graph.

development times. However, ANOVA revealed that the overall model was significant ($R = 0.385$), ($F(2,162) = 14.01$, $P < 0.001$). On closer inspection of the results, the variance inflation factor (VIF) values for the predictors were given as less than 10, and the tolerance (Tol.) values were above 0.1 (VIF = 1.169, Tol. = 0.856), and additionally both of these values were identical for both predictors; therefore, collinearity was not deemed a problem in this analysis (Lind et al., 2008).

The coefficients (beta values) and the corresponding *t*-values for the two predictors suggested strongly that the concentration of chromium VI has a greater predictive power for the nematode reproductive numbers than development times as indicated by the significance of the *t*-value for treatments ($P < 0.001$) against that for development times ($P > 0.05$).

Since infectivity is another valuable indicator of the effects of chromium VI on the behaviour of *S. feltiae*, this too was analysed with multiple regression analysis. Since progeny counts and development times are indicators that occur after infection, only survival times and treatments were tested as possible predictors of infectivity rates. This time, only 11% of the variation was explained by the predictors ($R^2 = 0.112$). Additionally, ANOVA revealed that the model was statistically not significant ($R = 0.335$), ($F(2,29) = 1.083$, $P > 0.05$).

3.4 Discussion

The focus on most nematode toxicity testing is on sublethal effects rather than mortality as they can obviously be more accurate and informative. In this study, sublethal end points, such as infectivity, reproduction potential and survival, were employed to detect the effects of chromium VI on the behaviour of the nematode. These end points have previously been employed as indicators of EPN fitness on laboratory-maintained strains to describe the effects of age (Yoder et al., 2004), field application methods in biological control programmes (Perez et al., 2004), temperature (Hazir et al., 2001) and the production methods (Grewal et al., 1999). The authors maintain that these end points can also be observed to describe the fitness of *S. feltiae* in compromised ecological habitats. These end points, being sublethal, plus the fact that the nematode appears to possess a reproductive mechanism that might allow it to survive for longer than other species in polluted soils, increase the chances of isolating *S. feltiae* in contaminated soils which can then be assessed for the effects of the contaminant. *Steinernema* species can be easily isolated and identified to genera level quite rapidly, and identified to species level if required. However, Griffin and Downes (1994) and Dillon et al. (1999) have described *S. feltiae* as a prevalent species in Ireland along with *S. affinis,* and isolation from soil is rarely difficult (personal observations).

Steinernema feltiae is parasitic and feeds and reproduces within suitable host insects, which die after

infection and usually before nematode reproduction (Boemare and Akhurst, 1990). The IJ is largely soil dwelling and is the only life-cycle stage that exists outside the host cadaver; it is a non-feeder and possesses a thick cuticle to survive extremes of moisture, UV rays and desiccation and to prevent the entry of pathogens (Poinar, 1990). Despite this, results presented in this paper suggest that it is still adversely affected by the presence of chromium VI when exposed to it in soils. What is interesting here is the increase in nematode progeny numbers in response to the presence of chromium VI.

In the literature, reports on the responses of nematode communities generally vary when describing the effects of heavy metals on these communities. Some studies describe the increase in nematode numbers, or nematode densities, under the presence of heavy metals (Weiss and Larink, 1991; Jaworska and Gorczyca, 2002; Gyedu-Ababio and Baird, 2006). Most of these studies describe observations where density increases in communities are a result of the ability of certain nematode species to take advantage of stressed environments, and the inability of others to do likewise. Other reports describe a general decrease in nematode biodiversity, but at relatively high heavy metal concentrations (Korthals et al., 1996). Others yet again describe the ability of nematode communities to significantly resist the effects of heavy metal pollution (Millward et al., 2001; van Vliet and de Goede, 2008).

The study presented here reports that the IJs of *S. feltiae* are adversely affected by relatively low concentrations of chromium VI in the soil in terms of extended survival of IJs and infectivity rates. On the other hand, although less host food larvae become infected with IJs as a result of protracted chromium VI exposures, numbers of resulting progeny increase with increasing chromium concentrations where successful infection does occur. There was also an observed increase in development times (the length of time between infection and death of host larvae to emergence of new IJ generations). It was unclear whether the increased development times were a direct result of the presence of chromium VI, or a response from *S. feltiae* to slow development in order to produce larger numbers of offspring where possible. Certainly, the recording of a non-significant Kendall's Tau-*b* correlation coefficient between survival times and development times suggests that regardless of how long IJs can remain biologically active in chromium VI contaminants, this will nevertheless have little effect on subsequent development times of successful IJs. Comparing this with the significant Kendall's Tau-*b* positive correlation coefficient between increasing chromium VI concentrations and development times, it appears that it will be the concentration of chromium VI rather than the duration of exposure (up to an approximate exposure duration of 5–6 days) that may slow development among nematodes that can successfully infect host insects. Related to this finding is the significant negative Kendall's Tau-*b* correlation coefficient achieved between concentrations of chromium VI and survival rates. This result suggests that increasing concentrations of chromium VI encourage reduced survival rates that in turn limit the number of available active IJs. However, a significant Kendall's Tau-*b* positive correlation coefficient was also achieved between progeny development times and nematode numbers, suggesting that the longer the development times, the higher the numbers of nematode progeny produced. The logical assumption therefore is that in order to produce more offspring, longer development times are required, regardless of the factors contributing to reduced development times or increasing numbers. Ultimately, the conclusion that can be drawn from these correlation tests is that the nematodes can survive in the presence of increasing concentrations of chromium VI up to concentrations of 100 ppm up to a period of 5–6 days, yet these survival rates are reduced, thus infectivity rates are reduced, and nematodes that do survive up to the 5- to 6-day cut-off point exhibit longer development times within hosts and produce significantly more offspring.

Either way, it appears that this organism possesses a mechanism to allow it to develop larger numbers of offspring when exposed to a chromium VI-contaminated environment in spite of adverse affects on the IJ pre-infection. This post-infection increase in reproduction may allow the nematode a greater chance of population recovery by:

1. Increasing progeny numbers that might enable at least some progeny to move out of a

contaminated area after emergence, either by themselves or by host mobility between IJ infection and death;

2. Increasing the chance of producing progeny that possess a higher degree of resistance to chromium VI; or

3. Allowing for increased progeny development times that may eventually discharge progeny into an environment with greatly reduced contaminant due to action from biological, chemical, physical or other environmental processes.

This last point is further supported when taking into account that an EPN-infected insect cadaver is considered a protective environment within which the nematode can feed, reproduce and develop in relative safety (Lewis and Shapiro-Ilan, 2002).

As interesting and as potentially useful as this attribute may be to both the nematode and the ecotoxicologist, it is also worth discussing it hypothetically in terms of genetic toxicology and what it really may signify for an *S. feltiae* population in a chromium VI-contaminated ecosystem. Genetic alterations among organisms existing in a polluted environment and the consequential population-genetic and evolutionary effects have been attracting the attention of ecotoxicologists in recent years (Deiter, 1993; Anderson et al., 1994; Bickham and Smolen, 1994; Depledge, 1994; Fox, 1995; Hebert and Luiker, 1996; Belfore and Anderson, 1998; Cronin and Bickham, 1998). It has been proposed that populations can be subject to an increase in genetic variability as a result of toxicant exposure (Bickham et al., 2000). Conversely, toxicants can reduce genetic variability because of population crashes and resultant loss of numbers creating a bottleneck effect (Guttman, 1994; Ellegren et al., 1997). Overall, these experiments recorded a total reduction in nematode progeny numbers per individual treatments when compared with the controls. This was due to less wax worms succumbing to IJs from 10 ppm of chromium VI up to 100 ppm, as illustrated by Fig. 2.3. The risk of a population/genetic bottleneck developing when the species encounters a contaminant in its environment appears to be quite high because, although there are significantly higher progeny numbers being produced when an infection event occurs, these events are occurring less in the presence of chromium VI. The nematodes emerging from a single cadaver are generally the offspring of a small number of original mating pairs that invaded the haemocoel of the insect larvae, especially in the natural environment as there may be less nematodes available than in laboratory experimental conditions. Therefore, they may possess a characteristically low-level genetic diversity. Organisms maintained in laboratories have been demonstrated to suffer from reduced fitness due to excessive inbreeding (Bilgrami et al*.,* 2006). A similar situation of excessive forced inbreeding would probably further reduce the ability of the nematode to survive long-term exposure to chromium VI due to the resulting general decrease in population or strain heterozygosity. Indeed, reduced heterozygosity has been associated in other organisms with decreased resistance to disease (Ferguson and Drahushchak, 1990), decreased growth rates (Wohlfarth, 1985) and decreased fertility (Leberg, 1990). On the other hand, an increase in progeny numbers might, theoretically, eventually increase the chances of genetic mutations occurring that may confer a competitive advantage on future generations of the strain or species in that polluted environment. Certainly, selection of strain-specific adaptations to environmental extremes has been recorded among EPN nematodes for host-finding ability (Gaugler et al., 1989), for desiccation (Griffin and Downes, 1994) and for temperature (O'Leary and Burnell, 1995). This suggests that there may be sufficient variability occurring within populations of EPN to expect positive responses to selection pressures.

Chapter 2 of this report described an increase in nematode progeny numbers and increased development times among nematodes that fed, reproduced and developed within heavily chromium VI-contaminated host cadaver larvae. This study did not take into account the behaviour of the IJs in external contaminated environments. The survival of the nematode and significantly increased progeny numbers were observed at extremely high chromium concentrations (up to 1,400 ppm). This differs markedly with the current study as nematodes are observed to be impaired after 10–20 ppm chromium VI exposure. The ability of the nematode to survive higher

in vivo contaminations may be due to a far higher rate of biological activity occurring within an infected host cadaver, and thus greater forces working on the decomposition of the contaminant. Sterile sandy soils are far less biologically active, although it is known that bioavailability of heavy metals in soils is reduced due to the binding of heavy metal salts to organic matter and clay (van Vliet and de Goede, 2008). However, soil nematodes are essentially aquatic organisms, employing moisture levels to aid mobility, and moist loose sandy soils, such as the type used in these experiments, may provide an increased surface area through which nematode IJs can come into contact with bioactive contaminants.

Another reason for increased nematode resistance to chromium in insect cadavers may be due to the action of the symbiotic bacteria on the contaminant. Bacteria are known to release metal-binding compounds in response to the presence of heavy metals (Bridge et al., 1999). However, bacterial populations are also known to decrease with increasing concentrations of chromium (Parameswari et al., 2009), although they may continue to be biologically active (Brown et al., 1994). This may help explain the increasing development times of *S. feltiae* in increasing concentrations of chromium VI. Additionally, Eagon (1984) and Vasanthy (2004) have described a higher tolerance of Gram-negative bacteria to heavy metals than Gram positives and this further supports evidence that the Gram-negative *Xenorhabditis* species symbiotic to *S. feltiae* may allow greater nematode resistance to heavy contamination in infected food cadavers.

As previously discussed, the nematode *C. elegans* is a model organism species and is one of the most widely studied nematodes in ecotoxicological research. It is deemed suitable because it can be bred easily, exhibits easy test performance and short duration of test, and it places few demands on materials and resources (Williams and Dusenbery, 1990; Donkin and Dusenbery, 1993; Peredney and Williams, 2000; Boyd and Williams, 2003a). Although these attributes are indeed useful, it is mostly used in laboratory-based studies because stocks are maintained easily and it is difficult to identify from soil samples (Kammenga and Riksen, 1996). Laboratory-based studies demonstrate clear causal relationships between contaminants and organisms because conditions can be controlled and there is often lower variability (Sochova et al., 2006). Laboratory-based approaches are an important first step to understand the consequences of environmental damage or disturbance, but the complexity of natural ecosystems would render it difficult to accurately relate effects observed in controlled laboratory environments to those that may be observed in the field. This fact is well recognised and other species have been investigated for potential as more relevant ecological test species in place of *C. elegans* such as *Plectus acuminatus* (Kammenga and Riksen, 1996) and *Panagrellus redivivu*s and *Pristionchus pacificus* (Boyd and Williams, 2003b) and *S. feltiae* (Boyle and Kakouli-Duarte, 2008). The findings presented in this paper further support the proposition of *S. feltiae* as a valuable addition to the canon of the biological ecotoxicologist to help further understand the effects of heavy metal contaminants on biota.

3.5 Conclusions

It is clear that chromium VI impairs the ability of *S. feltiae* to function normally. Survival, infectivity and the amount of time it takes for it to reproduce in contaminated environments are all adversely affected. Despite this, the clear increase in numbers emerging from infected cadavers suggests that this nematode possesses some manner of mechanism to increase its chances of survival in a compromised habitat.

The fitness of progeny produced from IJs exposed to chromium VI was not assessed in these experiments, depriving the study of vital insights into the behaviour characteristics of the next generations when released back into the contaminated environment. Additionally, DNA studies such as whole genome approaches such as random amplified polymorphism DNA (RAPD)-PCR, microsatellite studies or, indeed, comprehensive DNA expression analysis may provide valuable data that correspond with observations recorded in this paper. Information about genes expressed in the presence of chromium VI and recorded increases or reductions in genetic variability as a consequence of chromium VI pollution will allow the development of important and useful molecular markers that can be

employed in conjunction with fitness parameters to assess the ecological impact of contamination events.

Additionally, studies of the effects of other heavy metals, either individually or as combinations, on the sublethal end points assessed in this study will only serve to further the understanding of the effects of industrial contaminants on nematodes as they exist in complex natural ecosystems.

These results describe characteristic observations that are a further crucial step in developing the nematode *S. feltiae* as a biological ecotoxicological sentinel organism in Ireland.

4 Differential Gene Expression in *Steinernema feltiae* Nematodes in Response to Chromium VI Exposure in Contaminated Host Cadavers

4.1 Introduction

The effects of heavy metals and, indeed contaminants in general, on the molecular constitution of an organism have important implications for ecotoxicology, evolution, agriculture, food production, and livestock and human health, to name but a few. The nematode *C. elegans* has been investigated for the expression of genes in the presence of cadmium (Liao and Freedmand, 1998), for cadmium resistance (Cui et al., 2007) and arsenic response (Tseng et al., 2007). These studies often revealed genes that were homologous to genes of previously known function within higher organisms, or produced genes that were previously known in *C. elegans* but not known to be involved in toxicity responses. Furthermore, these types of studies often revealed novel genes that encoded novel proteins that had not previously been identified. These studies are crucial as many nematode genes and transduction pathways have high evolutionary conservation and contribute to an understanding of the functional roles of contaminants in higher organisms.

There are many molecular approaches to achieve the isolation of differentially expressed genetic material. One such approach is subtractive cloning. This is a powerful technique for isolating genes expressed in one cell population but not in another. It has origins in nucleic acid reassociation techniques, where the ability of the DNA double helix to denature and separate and then re-hybridise again is exploited. In a process called driver excess hybridisation, nucleic acid containing differently expressed material (target cDNA or [+] cDNA) is hybridised to complementary nucleic acid material believed to lack the sequences of interest (the driver or [–] cDNA).

The investigation of molecular expression of genes in *S. feltiae* nematodes reared in host cadavers heavily contaminated with chromium VI was conducted in order to create a valuable future resource for environmental toxicology, and specifically for chromium VI toxicology. This was achieved by creating a subtracted cDNA library of genes that were expressed in the presence of chromium VI in *S. feltiae*.

4.2 Materials and Methods

4.2.1 *Rearing of* S. feltiae *IJs (unexposed nematodes; driver [–] samples)*

Using standard 9-cm Petri dishes, the lids were lined with two sheets of Whatman No. 1 filter paper. A 1- to 1.5-ml volume of IJ (*S. feltiae* Strain 12(1)) suspension in water (containing approximately 200–1,000 nematode IJs) was applied until the paper was moist, but not overly wet. Ten *G. mellonella* (wax worms) were placed on the filter paper per Petri dish, and 10 Petri dishes were used in total. The Petri dish base was placed on top and sealed, and the plates were then incubated at 20°C for 4 days.

Parasitised larvae were placed on White traps, and treated as previously described. Harvesting was carried out every day after initial IJ emergence was observed (5–10 days after placing on White traps). This was performed by decanting the contents of the reservoir daily into an appropriately labelled centrifuge tube. The reservoir was replenished with fresh water as before and cadavers were further incubated as described. Nematodes were washed three times in 50-ml centrifuge tubes by allowing them to settle to a pellet after standing, removing the supernatant, and replacing with sterile double-distilled water. This was repeated as much as necessary until a clear supernatant was observed. The nematodes were then transferred to micro-centrifuge tubes and were stored in RNAlater (Sigma) at –70°C until required. The harvesting procedure was repeated until approximately 4 g of IJs were collected.

4.2.2 *Rearing of* **S. feltiae** *nematodes in chromium VI-contaminated cadavers (exposed sample, target or [+] samples)*

Last instar larvae of the wax moth *G. mellonella* were weighed to ensure that all larvae were in the 250- to 350-mg weight range. A total of 40 wax moth larvae were injected with approximately 10–15 IJs of *S. feltiae* Strain 12(1). After larval deaths, which occurred approximately 24 h after the injections, the cadavers were weighed again and the weights were recorded. Aqueous sodium dichromate was then injected into larvae to give a concentration of 400 ppm per cadaver.

Wax worm larvae were placed on White traps and harvested, washed and stored in RNAlater as described above.

4.2.3 *Extraction of total RNA*

Total RNA was isolated from both chromium VI-exposed and unexposed nematodes using a technique based on that described by Gauthier and Madison (1997). Two grams of fresh nematode pellet were frozen in liquid nitrogen and crushed in a sterile mortar with a pestle. A 1-ml volume of GTC solution (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol) was added. This mixture was removed to a sterile 50-ml polypropylene centrifuge tube and a further 9 ml of GTC solution were added. Then 2 ml of 2 M sodium acetate were added, followed by 6 ml of 0.1 M citrate buffer saturated phenol (pH 4.3). Additionally, 1 ml of chloroform/isoamyl alcohol (24:1) was added. The preparation was then incubated on ice for 10 min. It was then centrifuged at 10,000 × *g* for 10 min at 4°C. Total RNA separates into the upper aqueous phase after centrifugation and this was transferred to a 1.5-ml micro-centrifuge tube (approx. 500 μl per tube). Two volumes of 95% ethanol, pre-chilled at –20°C, were added to each micro-centrifuge tube and the solution was mixed by inversion. The samples were centrifuged at 10,000 × *g* for 10 min and ethanol decanted thereafter. A white pellet of RNA remained, which was washed with 70% ethanol. Once this ethanol was decanted, the pellet was air-dried at room temperature in a DNA-clean cabinet for 1 h. The samples were re-suspended in deionised, double-distilled water.

The purity and concentration of isolated RNA were confirmed by UV analysis at 260 and 280 nm, and for integrity using 1% agarose/formaldehyde gel electrophoresis (Promega RNA Notebook).

4.2.4 *Isolation of messenger RNA*

Messenger RNA (mRNA) was isolated from both driver and target samples using the PolyATtract mRNA Isolation System (Promega), following kit instructions.

The starting quantities of total RNA for each sample were 896 μg of total RNA for driver samples, and 980 μg of total RNA for target samples.

After mRNA was isolated, the quantities of each were 15 μg of mRNA for the driver samples and 17 μg of mRNA for the target samples.

4.2.5 *Generation of cDNA from mRNA driver [–] and target [+] samples and development of first-round libraries*

Both target and driver mRNA were used to generate cDNA libraries using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen), following kit instructions. Briefly, this involved generating first-, then second-strand DNA from the mRNA, followed by the addition of restriction enzyme-specific adapters (*Sal* I and *Not* I adapters) which were then used to ligate the cDNAs to pSPORT 1 vectors (Invitrogen) and transformation into MAX Efficiency DH5α™ *Escherichia coli* chemically competent cells (Invitrogen). A small quantity of newly transformed cells (100 μl from each transformation reaction volume of 1 ml) was plated on lysogeny broth (LB) agar with 100 μg/ml ampicillin and incubated overnight at 37°C to determine adequate transformation efficiency. Once determined, the remaining transformations were plated under identical conditions. Any remaining cDNA-pSPORT ligation reaction was also transformed and plated identically.

4.2.6 *Expansion of cDNA libraries*

All plates with colonies, and for both target [+] and driver [–] samples, were taken from the incubator once cell colonies had grown large enough to be confluent. For each sample group of plates, 1 ml of sterile LB broth was used to flush colonies from the plates. The resulting mixture was decanted into a 50-ml sterile centrifuge tube for each cDNA library (both for target

and driver libraries – care was taken not to cross-contaminate). Colony mixtures were mixed by gentle inversion and aliquoted into cryogenic tubes of 1.5-ml volumes; 1.5 ml of sterile 28% glycerol were added and the tubes were gently inverted for homogenisation. They were labelled and stored at –70°C for long-term storage.

4.2.7 Generation of subtracted cDNA library

The following procedure is based on that described in Ausubel et al. (2003). Modifications include the replacement of Lambda vectors with pSPORT plasmids and associated changes resulting thereof, and the separation of plasmid/vector using electrophoresis rather than employment of a sucrose gradient and centrifugation.

Samples from each stored target and driver cDNA library were defrosted on ice and used to inoculate 50 ml of LB for each library in sterile 50-ml centrifuge tubes with 100 μg/ml of ampicillin. They were incubated for 24 h at 37°C.

Plasmids were isolated from broth cultures using the StrataPrep® Plasmid MiniPrep Kit (Stratagene), following instructions provided. Plasmid extraction continued until 105 μg of plasmid were isolated for the driver cDNA library and 108 μg of plasmid were isolated for the target library.

Concentrations and purity of plasmid isolations were measured using UV spectrophotometric analysis at 260 and 280 nm. Integrity tests were performed using 1% gel electrophoresis.

Plasmids were separated from their respective inserts by digestion with restriction enzymes *Sal* I and *Not* I (Promega), following the manufacturer's instructions. Digestion reactions were stopped by adding 5 μl 0.5 M EDTA to each reaction and incubated for 10 min at 65°C.

cDNA and plasmids were purified using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifugation at 10,000 × *g* for 15 min. Upper aqueous phases were removed and placed into new sterile 1.5-ml centrifuge tubes. DNA was purified using the salt/ethanol technique as follows: a 9:1 ratio of cDNA/plasmid mix to 3 M sodium acetate was prepared and mixed by inversion. Two volumes of 95% molecular grade ethanol (–20°C) were then added. After centrifugation at 10,000 × *g* for 15 min, the pellets were washed with 70% ethanol. The pellets were then dried and reconstituted in 10 μl water.

The entire mixtures of target [+] cDNA and plasmid and driver [–] cDNA and plasmid were separated using 1% agarose (with 0.5 μg/ml ethidium bromide) electrophoresis. The entire sample volumes for each target [+] and driver [–] were added to a separate well each. An electric current was applied at 60 V for 2 h.

Using a transilluminator, separation of cDNA inserts and plasmids was confirmed and inserts were cut from the agarose gel using a scalpel and a transilluminator, and were then purified from the resulting gel fragments using the Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions.

The *Not* I and *Sal* I adapters were removed from the driver [–] cDNA by digesting with S1 nuclease (Sigma–Aldrich), according to the manufacturer's instructions. The cDNA was then purified using the phenol/chloroform/isoamyl alcohol and the salt/ethanol technique, as described above. This cDNA was further treated by digesting with *Rsa* I and *Alu* I restriction enzymes (Promega) according to the manufacturer's instructions. These two procedures ensure that small blunt-ended fragments of driver [–] cDNA were achieved. Once again, the cDNA was then purified using the phenol/chloroform/isoamyl alcohol and the salt/ethanol technique, as described above.

Both the digested driver [–] cDNA and the intact target [+] cDNA were then hybridised as follows: a mixture of both cDNAs was prepared to give a 49:1 ratio of driver [–] cDNA to target [+] cDNA. This was prepared in a final volume of 5.5 μl, which was used in the following reaction mixture: 12.5 μl deionised formamide (Sigma) in a 50:50 v/v final; 6.25 μl 20 × SCC; 0.25 μl of 1 M $NaPO_4$ (prepared by adding 1 M NaH_2PO_4 to 1 M Na_2HPO_4 until solution pH = 7); 0.25 μl 1 M EDTA; 0.25 μl 10% SDS; 0.5 μl 10 mg/ml yeast tRNA (0.2 mg/ml final). This mixture was homogenised by vortexing, collected by brief centrifugation (10 s at 2,000 × *g*) and placed into a boiling water bath for 5 min to separate the cDNA double helix to facilitate

hybridisation. The mixture was then briefly centrifuged as previously and then incubated at 37°C for 24 h.

A 200-μl volume of TE buffer was added to the mixture and then mixed by inversion and brief centrifugation (10 s at 2,000 × *g*); 225 μl of phenol/chloroform/isoamyl alcohol (25:24:1) were added and the mixture was extracted by gentle inversions for 5 min. The tube was then centrifuged at maximum speed on a microcentrifuge (12,000 × *g*) for 1 min to separate the phases. The upper phase was removed to a new microcentrifuge tube and 225 μl of chloroform/isoamyl alcohol (24:1) were added and the mixture extracted and centrifuged as before.

The upper aqueous phase was removed and placed into a new microcentrifuge tube and the cDNA mixture was purified using the salt/ethanol procedure as described above. The resulting hybridised and purified cDNA was then re-ligated to pSPORT plasmid vectors from the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen), following the kit instructions. This time only cDNA fragments with both *Sal* I and *Not* I adapters (hybridised fragments that are present only in the cDNA that was extracted from nematodes exposed to chromium VI) will ligate to the vector, which has *Sal* I and *Not* I ligation sites. The entire ligation mixture of cDNA/plasmid DNA was transformed into MAX Efficiency DH5α™ *E. coli* chemically competent cells (Invitrogen), following the supplier's instructions.

All transformed cells were plated onto LB agar with 100 μg/ml ampicillin that had previously been spread with 40 μl Blue White Screening Reagent (Sigma). This contained 40 mg/ml IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in DMSO (dimethylsulfoxide). Plates were incubated at 37°C for 36 h.

Positive recombinant colonies were identified as white colonies (as opposed to blue non-recombinant colonies) and transferred to tubes of LB with 100 μg/ml of ampicillin and incubated at 37°C for 36 h. Aliquots from these cultures were then mixed 50:50 with 28% sterile glycerol, numbered, and stored at –80°C as stock cultures.

The remainder of the culture solutions were used to extract the plasmids from the transformed cells in order to sequence the cDNA inserts for identification. Plasmids were extracted using the StrataPrep® Plasmid MiniPrep Kit (Stratagene), following instructions provided. Plasmid/Insert extracts were digested with *Sal* I and *Not* I restriction enzymes (Promega) to remove inserts from plasmids, following the manufacturer's instructions, and visualised using 1% agarose electrophoresis (with 0.5 μg/ml ethidium bromide) and a transilluminator. This helped identify which transformed colonies were true transformants with actual cDNA inserts. From these results, sample colonies were chosen for DNA sequencing based on presence of cDNA inserts on the gels. Inserts were subsequently sequenced by a contract sequencer (Qiagen, Germany) and stored as data on computers. DNA sequences were analysed using the Basic Local Alignment Search Tool (BLAST), ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/), and the ExPASy website (http://www.expasy.ch).

4.3 Results

A total of 300 putative positive recombinants were achieved after the final ligation and transformation step, 96 of which were chosen to be sequenced on the basis of the *Sal* I/*Not* I digestion of the cDNA insert/plasmid complexes. Of these, 16 samples returned with positive reads, indicating that these plasmids contained inserts of cDNA (Fig. 4.1). Analysis of these sequences indicates that four gene families are represented in the subtracted library, as well as a group of genes that have no known function and were believed to be novel. Five recombinant colonies contained genes encoding for glutamine synthetase, four more such colonies contained sequences for thioredoxin genes, two colonies contained genes that appear to be involved in nitrate assimilation and one colony contained sequences that appear to code for the N^{pro} gene regions of the bovine viral diarrhoea (BVD) virus (Fig. 4.1). There were three colonies that contained sequences that appear to code for genetic material of unknown function.

Transformed Colony Number 148 B

<u>Glutamine synthetase gene fragment.</u>

**AAAAATCGGATATGATTTTAAAACCTGATGTTAAAAGTGCTTTTTTAGATCCATTTACAGCAGATCCTACTATCATAGTAATTTGTGAT
GTATATGATATTTACAAAGATCAAATGTATGAAAAATGTCCAAGAAGTATTGCCAAAAAAGCAATGCAACACTTAAGTACAAGCAAT
ATCGCTGACACTGCTTATTTTGGTCCTGAAAATGAATTTTTTATTTTTG**

Transformed Colony Number 151 A

<u>Glutamine synthetase gene fragment.</u>

**CGTTTTTAGATCCTTTTACAGCAGATCCTACTATCATAGTAATTTGTGATGTATATGATATTTACAAAGATCAAATGTATGAAAAATGT
CCAAGAAGTATTGCCAAAAAAAGCAATGCAACACTTAAGTACAAG**

Transformed Colony Number 152 B

<u>Glutamine synthetase gene fragment.</u>

**AAATCAGATATGATTTTAAAACCTGATGTCGAAAGTGCGTTTTTAGATCCTTTCACAGCTGATCCTACCATAATAGTAATTTGCGATGT
ATATGATATTTATAAAGAACAAATGTATGAAAAATGTCCAAGAAGTATAGCAAAAAAAGCTATGCAATATTTATCACAAAGCAATATA
GCAGATAAGGCTTATTTTGGTCCTGAAAATGAATTTTTTATTTTTGATAATGTAAAAATAGTTGATTCTTCTAATTGTGCAAAATATGAA
GTAGATACAGAAGAAGGTGAGTGGA**

Transformed Colony Number 155 B

<u>Glutamine synthetase gene fragment</u>

**AAATCGGATATGATTTTAAAACCTGATGTTAAAAGTGCTTTTTTAGATCCATTTACAGCAGATCCTACTATCATAGTAATTTGTGATGT
ATATGATATTTACAAAGATCAAATGTATGAAAAATGTCCAAGAAGTATTGCCAAAAAAGCAAT**

Transformed Colony Number 157 A

<u>Significant homology to bovine viral diarrhoea virus</u>

**AGTGGTGAGTATACCCCGCCTGTGAAATCCTCCTTGGCGAAGGCCGAAAAGAGGCTAACCATGCCCTTAGTAGGACTAGCAAAATA
AGGGGGGTAGCAACAGTGGTGAGTTCGTTGGATGGCTGAAGCCCTGAGTACAGGGTAGTCGTCAGTGGTTCGACGCTTTGGAGGAC
AAGCCTCGAGATGCCACGTGGACGAGGGCATGCCCACAGCACATCTTAACCTGGACAGGGGTCGTTCAGGTGAAAACGGTTTAACC
AACCGCTACGAATACAGTCTGATAGGATGCTGCAGAGGCCCACTGTATTGCTAGTGGTGAGTATACCCCGCCTGTGAAATCCTCCTT
GGCGAAGGCCGAAAAGAGGCTAACCATGCCCTTAGTAGGACTAGCAAAATAAGGGGGGTAGCAACAGTGGTGAGTTCGTTGGATG
GCTGAAGCCCTGAGTACAGGGTAGTCGTCAGTGGTTCGACGCTTTGGAGGACAAGCCTCGAGATGCCACGTGGACGAGGGCATGC
CCACAGCACATCTTAACCTGGACAGGGGTCGTTCAGGTGAAAACGGTTTAACCAACCGCTACGAATACAGTCTGATAGGATGCTGC
AGAGGCCCACTGTATTGCT**

Transformed Colony Number 160

<u>Thioredoxin gene fragment</u>

**TGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATACCATGAG
CGGTAAGCCAATTCCAAATCCACTCCTAGGCCTCGATAGTACTGCCATGGGCAGCACCTGGGTGCTGGTGGGCGGAGTCCTTGCAG
CTCTGGCCGCGTATTGCCTGACAACAGGCAGTGTGGTCATTGTGGGTAGGATTATCTTGTCCGGGAGGCCGGCCATTGTCCATATGG
GAGGCGGATCGGGAGGAGGCGGAGGATCTGATAAAATTATTCATCTGACTGATGATTCTTTTGATACTGATGTACTTAAGGCAGAAG
GTGCAATCCTGGTTGATTTCTGGGCACACTGGTGCGGTCCGTGCAAAATGATCGCTCCGATTCTGGATGAAATCGCTGACGAATATC
AGGGCAAACTGACCGATGCAAAACTGAACATCGATCACAACCCGGGCACTGCGCCGAAATATGGCATCCGTGGTA**

Figure 4.1. DNA sequences of gene fragments present in positive recombinant colonies from the chromium VI subtracted cDNA library. Sequences were identified (where possible) using the Basic Local Alignment Search Tool (BLASTn; 'n' denotes nucleotide BLAST) in GenBank.

Transformed Colony Number 166

Thioredoxin gene fragment

CTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGT
TGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTAT
GGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGA
CGTGGTTTTCCTTTGAAAAACACGATGATAATACCATGAGCGGTAAGCCAATTCCAAATCCACTCCTAGGCCTCGATAGTACTGCCAT
GGGCAGCACCTGGGTGCTGGTGGGCGGAGTCCTTGCAGCTCTGGCCGCGTATTGCCTGACAACAGGCAGTGTGGTCATTGTGGGTA
GGATTATCTTGTCCGGGAGGCCGGCCATTGTCCATATGGGAGGCGGATCGGGAGGAGGCGGAGGATCTGATAAAATTATTCATCTG
ACTGATGATTCTTTTGATACTGATGTACTTAAGGCAGAAGGTGCAATCCTGGTTGATTTCTGGGCACACTGGTGCGGTCCGTGCAAAA
TGATCGCTCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAACTGACCGATGCAAAACTGAACATCGATCACAACCCGGGC
ACTGCGCCGAAATATGGCATCCGTGGTATC

Transformed Colony Number 168

Thioredoxin gene fragment

CACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAAGAGTCAAATGGCTCTCCTCAAGCGTATT
CAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTA
GTCGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATACCATGAGCGGTA
AGCCAATTCCAAATCCACTCCTAGGCCTCGATAGTACTGCCATGGGCCTGACAACAGGCAGTGTGGTCATTGTGGGTAGGATTATCT
TGTCCGGGAGGCCGGCTATTGTTCCCGACAGGGAGCTTCTCTACCAGGAGTTCGATGAAATGGAAGAGTGCCATATGGGAGGCGGA
TCGGGAGGAGGCGGAGGATCTGATAAAATTATTCATCTGACTGATGATTCTTTTGATACTGATGTACTTAAGGCAGAAGGTGCAATC
CTGGTTGATTTCTGGGCACACTGGTGCGGTCCGTGCAAAATGATCGCTCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAA
CTGACCGATGCAAAACTGAACATCGATCACAACCCGGGCACTGCGCCGAAATATGGCATCCGTGGTA

Transformed Colony Number 169

Thioredoxin gene fragment

TCGATAGTACTGCCATGGTTCCCGACAGGGAGCTTCTCTACCAGGAGTTCGATGAAATGGAAGAGTGCCATATGGGAGGCGGATCG
GGAGGAGGCGGAGGATCTGATAAAATTATTCATCTGACTGATGATTCTTTTGATACTGATGTACTTAAGGCAGAAGGTGCAATCCTG
GTTGATTTCTGGGCACACTGGTGCGGTCCGTGCAAAATGATCGCTCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAACTG
ACCGATGCAAAACTGAACATCGATCACAACCCGGGCACTGCGCCGAAATATGGCATCCGTGGTATCCCGACTCTGCTGCTGTTCAA
AAACGGTGAAGTGGCGGCATCCAAAGTGGGTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAAC

Transformed Colony Number 170

No known function ñ novel gene fragment

CTAGGGAATTAGCGGCCGCTAATTCCCTAGAGGATCCTTATTACTCCTTCGATCTCAAAGACTCCTGAAGGTTTGTGGACAGCGAAA
AGGATCTCATGATCTCTGCTCTGACGACCTCCCAGGCGCATGGCGAGTACTTTTTCTCCTTCAGGTACAATGTAATGCGCTGGAAGT
ACTTTCTAACTGCCAGGATGGAATCCTCCTTCATCAAAGGGGTTTCTGTAACGCCGACACCTTGGATCACGCATGCCTCCAGGTCGT
TCAGTTGCTGATACAACTCAGTGTAAAATTTATCCAGCAAAGTCTCATCCCAAGCTGCCGAGGAGTCCTTCGTAGAGAACAGGTTAA
AAATCTGCTGAATCATCTCGTGCAGCACAGGGATTGTTTCTGCTTTCTGAAACTGGTTTCCAAATTCCTCCTGCGGGAAGCCAAAGTC
GTGTCTGTCCTTCAAACACGAAAACAGAGAGATCTTTCTCATCTGAGCCAACAGCATCAGGGTTCTGCGCGAGCCCAGGGAGTGTGT
CTGTGGCAAATCACAGCCCAAGGCAGCTAGCGGCAAGAAACACAGCACTAGTATC

Transformed Colony Number 173

Some homology to nitrate assimilation genes ñ could be novel gene fragment

GCATGATAAGGGTAAAGTAATTTATTACTTGGATCATCCTGGTCAACGTTTTGCAATTGCTTGACTACACACTTACAGAATTCATGAG
ATCCCTGCTGATACTAGTGCTGTGTTTCTTGCCGCTAGCTGCCTTGGGCTGTGATTTGCCACAGACACACTCCCTGGGCTCGCGCAG
AACCCTGATGCTGTTGGCTCAGATGAGAAAGATCTCTCTGTTTTCGTGTTTGAAGGACAGACACGACTTTGGCTTCCCGCAGGAGGA
ATTTGGAAACCAGTTTCAGAAAGCAGAAACAATCCCTGTGCTGCACGAGATGATTCAGCAGATTTTTAACCTGTTCTCTACGAAGGA
CTCCTCGGCAGCTTGGGATGAGACTTTGCTGGATAAATTTTACACTGAGTTGTATCAGCAACTGAACGACCTGGAGGCATGCGTGAT
CCAAGGTGTCGGCGTTACAGAAACCCCTTTGATGAAGGAGGATTCCATCCTGGCAGTTAGAAAGTACTTCCAGCGCATTACATTGTA
CCTGAAGGAGAAAAAGTACTCGCCATGCGCCTGGGAGGTCGTCAGA

Figure 4.1 ***contd.***

Transformed Colony Number 176

No known function ñ novel gene fragment

**CAGATTTTAATCTTTCGGCAAAATTTAGATAAAAAATTTGGGTATTCGTATTTAGTTTCCAATATTATTTAGTTATACAAAACTATTGTC
TCAATAGACTGGCGTTGTAATGAGTAGTGTTCCCCTCGACTCTAGGGAATTAGCGGCCGCTAATTCCCTAGAGGATCCTTATTACTCC
TTCGATCTCAAAGACTCCTGAAGGTTTGTGGACAGCGAAAAGGATCTCATGATCTCTGCTCTGACGACCTCCCAGGCGCATGGCGAG
TACTTTTTCTCCTTCAGGTACAATGTAATGCGCTGGAAGTACTTTCTAACTGCCAGGATGGAATCCTCCTTCATCAAAGGGGTTTCTGT
AACGCCGACACCTTGGATCACGCATGCCTCCAGGTCGTTCAGTTGCTGATACAACTCAGTGTAAAATTTATCCAGCAAAGTCTCATC
CCAAGCTGCCGAGGAGTCCTTCGTAGAGAACAGGTTAAAAATCTGCTGAATCATCTCGTGCAGCACAGGGATTGTTTCTGCTTTCTG
AAACTGGTTTCCAAATTCCTCCTGCGGGAAGCCAAAGTCGTGTCTGTCCTTCAAACACGAAAACAGAGAGATCTTTCTCATCTGAGC
CAACAGCATCAGGGTTCTGCGCGAGCCCAGGGAGTGTGTCTGTGGCAAATCACAG**

Transformed Colony Number 177

Some homology to nitrate assimilation genes (could be a novel fragment)

**CGGATCGGCGTCTTTCGTCCCGAAAATGGGGCACTATCTTATCAGTTCGGTTCGAAAAACCCCGAGGCCACCCGAGTGGACGATAA
GCATGATAAGGGTAAAGTAATTTATTACTTGGATCATCCTGGTCAACGTTTTGCAATTGCTTGACTACACACTTACAGAATTCATGAG
ATCCCTGCTGATACTAGTGCTGTGTTTCTTGCCGCTAGCTGCCTTGGGCTGTGATTTGCCACAGACACACTCCCTGGGCTCGCGCAG
AACCCTGATGCTGTTGGCTCAGATGAGAAAGATCTCTCTGTTTTCGTGTTTGAAGGACAGACACGACTTTGGCTTCCCGCAGGAGGA
ATTTGGAAACCAGTTTCAGAAAGCAGAAACAATCCCTGTGCTGCACGAGATGATTCAGCAGATTTTTAACCTGTTCTCTACGAAGGA
CTCCTCGGCAGCTTGGGATGAGACTTTGCTGGATAAATTTTACACTGAGTTGTATCAGCAACTGAACGACCTGGAGGCATGCGTGAT
CCAAGGTGTCGGCGTTACAGAAACCCCTTTGATGAAGGAGGATTCCATCCTGGCAGTTAGAAAGTACTTCCAGCGCATTACATTGTA
CCTGAAGGAGAAAAAGTACTCGCCATGCGCCTGGGAGGTCGTCAGAGCAGAGATCATGAGATCCTTTTCGCTGTCCACAAACCTTC**

Transformed Colony Number 224

No known function ñ novel gene fragment.

**CTATGACGTCGCATGCACGCGTACGTAAGCTTGGATCCTCTAGAGCGGCCGCCCCTTTTTTTTTTTTTTTTTGAGAACTTGGCATCTC
CGCCAAGTCGAATTTATTCAATAAAAAATATAACAACAACAAAAAAAAGTTATTTTTCCTTCGTCTTCCGGTTTCCGCCCACTTCCATC
TT
CTCCAGCTCCTTATCCTTCCATTTGTCCATCGATACCGAGCGAACGAAGCTGACGTGGACTCCGAGGCCACGGTGGAGACCCGAGC
ATTCGAGGCAAATCCAGATGCCGTAAGTGACGCT**

Transformed Colony Number 225

Glutamine synthetase gene fragment

**GTTTTTAGATCCTTTTACAGCTGATCCTACTATCATAGTAATTTGCGATGTATATGATATTTACAAAGATCAAATGTATGAAAAATGTC
CAAGAAGTATTGCTAAAAAAGCAGTGCAACACTTAAGCACAAGCAATATCGCTGATACTGCTTATTTTGGTCCTGA**

Figure 4.1 *contd.*

The five glutamine synthetase gene fragments displayed very high similarity with each other, when analysed using ClustalW, supporting the hypothesis that they are representative of gene copies of this gene.

Similarly, the four thioredoxin fragments displayed high similarity, as did the two gene fragments for nitrate assimilation. The gene fragment that appeared to display homology to the N^{pro} gene regions of the BVD virus displayed homology to no other gene fragments sequenced in these experiments. Among the three unknown gene fragments, two fragments (from Colony Numbers 170 and 176) appeared to be identical to each other after ClustalW alignment experiments, but the third fragment (Colony Number 224) displayed no similarity to the other two, and also to none of the other sequences for the other gene fragments.

Therefore, represented thus far in this subtracted

library are: glutamine synthetase genes (five copies), thioredoxin genes (four copies), nitrate assimilation genes (two copies), sequences that appear to code for the N^{pro} gene regions of the BVD virus (one copy), novel genes of unknown function type I (two copies), and a novel gene of unknown function type II (one copy).

4.4 Discussion

The use of Blue–White Select™ Screening Reagent allows the user to differentiate between transformation of colonies with plasmids without ligated DNA inserts and those that transform with plasmids with inserts. Transformed colonies with plasmids that have inserts develop as white colonies. This is a result of disruption of the β-galactosidase gene by insertion of the DNA fragment into the vector's multiple cloning site, and that results in the loss of functional β-galactosidase activity on the β-galactosidase chromogenic substrate, X-Gal, and the inducer, IPTG. Colonies/Plaques bearing an insert containing vectors will remain white and may be easily distinguished from those bearing an intact cloning vector, which will be blue in colour. On the basis of this technique it was possible to distinguish which recombinant colonies putatively held cDNA inserts and which ones did not.

As discussed in the previous two chapters of this report, *S. feltiae* appears to possess some mechanism that allows it to survive in the presence of chromium VI when present in its environment. This mechanism is reflected in the genes uncovered in this series of experiments. Glutamine synthetase is a key enzyme involved in nitrogen metabolism in plants and animals (Stadtman and Ginsburg, 1974). It achieves this by catalysing the condensation of glutamate and ammonia to form glutamine. Glutamine and glutamate (glutamic acid) are amino acids known to be involved in maintaining a healthy immune function, and indeed are also important components in protein and nucleic acid synthesis. Glutamine has a well-established role in the treatment of serious injury and illness and there is strong evidence to suggest a role in the reduction of pro-inflammatory responses in cells as a result of the presence of reactive oxygen species (Muniandy et al., 2008). Furthermore, evidence suggests that glutamine synthetase is up-regulated in organisms exposed to contaminants and may play a crucial role in resistance to such environmental stressors (Boutet et al., 2004; David et al., 2005; Tanguy et al., 2005). Although trace chromium is an essential micronutrient required to promote the action of insulin in living cells allowing a physiological system to utilise sugars, proteins and fats, chromium VI is a known cellular toxicant that results in the formation of reactive intermediates and induces oxidative stress and oxidative tissue damage (Shrivastava et al., 2002). Chromium VI is reduced by glutathione in cells (Aiyar et al., 1991), which is a powerful antioxidant protecting cells from free radicals (Pompella et al., 2003). Glutathione is produced from glutamic acid and, therefore, for an upsurge of glutathione production to take place, it may be that an increase in glutamic acid must be present. A requirement for an increase in glutamic acid may consequently result in an increase in glutamine production, which may increase the energy available to the cells and, hence, increase the expression of glutamine synthetase. Glutamine is also an important factor in the inhibition of cell apoptosis (Fumarola et al., 2001), which can be a significant threat (Blankenship et al., 1994) to organisms exposed to chromium VI.

It therefore appears possible that the presence of glutamine synthetase genes in a chromium VI-subtracted cDNA library is a result of their increased expression due to the actions of reactive oxygen species produced in response to exposure to chromium VI within the nematode cells.

The presence of nitrate assimilation genes is similarly linked. As glutamine synthetase is an important regulator of nitrogen metabolism in a cell it is probably not surprising that DNA sequences with a homology to genes involved in nitrate assimilation are expressed in the nematode under toxic stress. What is unusual about their presence is that nitrate assimilation gene clusters have so far only been identified in fungi, algae and plants. It is unlikely that this nematode was actively assimilating pure nitrogen from the environment, and the genes expressed are most likely to be evolutionary derivatives, once shared by a common ancestor. The nematodes in this experiment were exposed to chromium VI during the organism's development and feeding phase in vivo, so an excessive intake of

nitrogen sources might have been achieved as a result of chromium VI being present.

The expression of thioredoxin genes within this nematode is perhaps the least surprising result. These genes are part of the well-known thioredoxin system, formed by the redox protein thioredoxin (TRX) and the enzyme thioredoxin reductase (TR). This complex is conserved throughout all species, from archaebacteria to humans (Holmgren, 1985). Thioredoxin reductase catalyses the reduction of TRX, in conjunction with hydrogenated nicotinamide adenine dinucleotide (NADH). It plays a role in many important biological processes such as selective redox regulation of some transcription factors, DNA synthesis through ribonucleotide reductase, extra cellular growth factor activity, protein disulfide reduction and protection against oxidative stress (Powis and Montfort, 2001; Lillig and Holmgren, 2007). Additionally, it has an important role in detoxification processes (Watson et al., 2004).

The most unusual genetic material (at this point) to be uncovered in this experiment was a gene that demonstrated homology to the BVD virus. It is probable that these sequences represent a conserved gene found across all taxa. For this to be the case, its function must be important enough to be universally required by primitive viruses through to nematodes at least. It is not known if these genes are involved with environmental stressors. However, there are some clues when the literature is reviewed. The fragment sequence appeared to display homology to the N^{pro} gene section of the BVD gene. This gene is putatively involved in the inhibition of cellular antiviral activity (Ruggli et al., 2003). This antiviral activity is in the form of interferon production by virally infected cells, and it is known to inhibit viral replication by interfering with protein synthesis. If the N^{pro} gene is involved in viral resistance to cellular antiviral mechanisms it could be that sections of this region have been conserved during evolution by incorporation into other gene systems, and continued with other similar detoxifying functions, such as disarming molecular contaminants. The N^{pro} gene is confined to viral species within the viral family, Flaviviridae, and has not been detected in other viral families (Tratschin et al., 1998). The gene fragments sequenced in these experiments were 569 bp and 695 bp, respectively, yet only an approximate 286-bp region demonstrated homology to the BVD viral genomes. This suggests that the gene may have been conserved in sections where the region involved in detoxification was incorporated into other genes that would have similar roles in higher organisms.

The possible presence of two previously undescribed gene groups from *S. feltiae* that are involved in responses to chromium VI provide strong potential for future work or projects. BLASTn database searches revealed no hits of significance, providing evidence that their function is unknown. Since the library generation procedure is designed to isolate only those genes that are expressed (i.e. activated), it is unlikely that they code for non-functional DNA regions. Identifying and isolating novel genes were beyond the scope of this project. However, it is important that these genes are identified and characterised in the future in order to benefit from the full potential of knowing about the genomic effects of chromium VI on various organisms.

The development of a subtracted cDNA library, enriched for genes involved with toxicological responses to chromium VI, confirms that the metal influences the expression of multiple genes in this nematode. Since only 96 of the recombinant colonies generated were actually sequenced, due to cost limitations, there is also the potential to discover even more genes influenced by chromium VI. The materials contained within this library can further benefit studies that determine the effects of chromium on the promoters of chromium-responsive genes and investigations that focus on the expression levels of these genes in response to varying levels of chromium VI. The latter can be achieved relatively easily by designing PCR primer systems that target the described genes and amplify them from nematodes further exposed to chromium VI, which can then be analysed using quantitative real-time PCR for expression levels. The former would be more extensive, but no less informative.

5 General Discussion

Organisms, as they exist in the environment, are constantly threatened by contaminants and pollutants. Their responses to such threats are varied and very much dependent on a myriad of chemical, biological and physical co-factors, such as pH, temperature, humidity, contaminant type and concentration, and the presence of other inhibiting or protective agents (Batzias and Siontorou, 2008). In addition, the effectiveness of an organism's own biological mechanisms which have evolved to deal with such threats plays a crucial part (Lasat, 2002; Falik et al., 2005). Some of these mechanisms are more effective than others from organism to organism. Indeed, an organism's life strategy and feeding mechanisms may be predictive as to how well it can survive a pollution event (Bongers, 1990; Millward et al., 2001).

There were marked differences in the survival rates of *S. feltiae* between exposure to chromium VI during in vivo development (Chapter 2) and IJ exposure in the soils (Chapter 3). The nematode appeared to be considerably more resistant to chromium VI when it is present in the food cadavers, which was an unexpected result. It may be because the food cadaver is highly biologically reactive. This may serve to reduce the contaminant to less toxic species, or neutralise it faster than it would in external soils. It may also be that the thick viscous nature of decaying tissues within the cadaver reduces the bioavailability of the toxicant to the nematodes. The nematode appeared to be affected by the chromium in both cases, however. Increases in progeny numbers were evident in both experimental approaches up to certain chromium VI concentrations and development times of progeny were increased (slowed). The testing of chromium on IJs proved the more informative of the two approaches, since it allowed measurement of infectivity and survival times of non-feeding IJs in contaminated soils in conjunction with those measured when analysing the effects of chromium VI in vivo (reproductive potential, development times).

β-Tubulin genes proved to be relatively ineffective at providing measurable molecular changes as a response to chromium VI. Changes that did occur were not site specific and were therefore unpredictable. Furthermore, most of these chromium VI-induced DNA variations occur on variable codon sites, thereby having no effect on the transcribed amino acid sequences. There is potential to employ quantitative real-time PCR analysis to determine the levels of gene expression or, indeed, for melting curve analyses measuring the increase in general DNA variations in the β-tubulin genes from nematodes exposed to contaminants and those that have not been exposed to contaminants (controls).

Subtracted cDNA libraries, or differential gene expression analysis, provide more promising platforms from which to investigate molecular contaminant responses. Significant work has already been carried out into the expression of cadmium-regulated transcription profiles using *C. elegans* (Liao and Freedman, 1998; Liao et al., 2002; Cui et al., 2007). Differential display analysis techniques are also extremely useful in understanding the effects of contaminants in other organisms (Liao and Freedman, 2002). Since biochemical pathways and biological molecular systems are generally conserved, information on the effects upon different invertebrate systems can serve to enhance the understanding of similar processes in higher organisms.

This project has resulted in the generation of a subtracted cDNA library that contains gene fragments representative of the molecular constitutional response of *S. feltiae* to chromium VI. Some of these genes have provided strong evidence that they belong to gene groups that are known to be involved in stress response. On the other hand, evidence suggests that some of the genes isolated are novel and have no published homologues. Furthermore, due to time and financial constraints, the remainder of the library has not been screened. There is evidence that there are more genes in this library (actually it is more than likely that there are at least 20–30 more) and future projects will take up this work and complete the screening of the genes present. The characterisation and description of

novel genes expressed in the presence of chromium VI represent an exciting challenge that may bolster the drive for the development of heavy-metal-specific molecular markers, which is currently under way in the molecular ecology and nematode research group at the Institute of Technology Carlow.

5.1 Conclusions

- The behaviour of *S. feltiae* is affected by the presence of chromium VI during development. However, chromium VI needs to be in relatively high concentrations for it to have an adverse influence. The nematode can increase progeny production in the presence of chromium VI concentrations up to 1,000 ppm, after which there are observable reductions in offspring production. This production ceases entirely after 1,800 ppm of chromium VI (Boyle and Kakouli-Duarte, 2008).

- The behaviour of *S. feltiae* is adversely affected by the presence of chromium VI in soils. It is affected by concentrations far lower than those that the nematode can resist in host cadavers (10–20 ppm). However, those nematodes that survive prolonged exposure to chromium VI in soils and successfully infect host cadavers were observed to produce far higher progeny numbers than those from uncontaminated soils. Development times were also observed to increase as a result of chromium VI exposures, and infectivity was observed to reduce markedly. Indeed, IJ survival rates, which are directly related to IJ infectivity rates, were also significantly reduced as a consequence of chromium VI exposure (Boyle et al., 2009).

- The two conclusions above suggest that a population mechanism is present in this nematode species to allow the production of larger numbers of progeny than normal in a contaminated environment. This mechanism appears to attempt to correct a potential shortfall in population numbers as a result of a reduction of viable infective parents by increasing the progeny numbers produced from those parents that do infect and mate successfully. This mechanism may also serve to increase the chances of selective pressures producing offspring that might exhibit higher resistance to environmental toxins than did their parents. Increased development times may also serve to allow external environmental factors, such as temperature, pH, wet/dry cycles and natural soil chemical and biological processes, to reduce the amount of available contaminants to later progeny.

- Chromium VI influences a range of gene types within *S. feltiae*. Here, six gene groups have thus far been partially observed, four of which have been shown to have demonstrated significant similarity to genes previously identified in other organisms. Two gene groups appear to have no significant matching similarities. All gene groups with similarities to the sequences elucidated in these experiments have known functions within cellular stress responses. One gene, the N^{pro} gene, appeared to have no direct involvement in cellular toxicological response, but there are similarities to a region involved in toxin resistance in viruses. Two more gene groups, thioredoxin and glutamine synthetase, are widely known to have roles in toxicological response, and another group, the nitrate assimilation gene group, can be shown to have links to contaminant resistance or removal when reviewing scientific literature.

- A principal deliverable was the development of RFLP and real-time PCR molecular markers for the detection of chromium VI-induced damage in β-tubulin genes. As reported in Chapter 2, the variations induced by chromium VI in these genes are too unpredictable and variable for RFLP or real-time PCR markers to be generated. Both of these techniques required DNA base-pair targets that are fixed (i.e. occur on the same DNA sections or codes so that enzymes or primers can target them). Therefore, it was decided that a subtracted cDNA library for genes expressed as a result of chromium VI exposure was a far more viable approach, and indeed the results described above demonstrate this. It was further hoped that Year 3 would allow for the characterisation of some of the chromium-expressed genes but, unfortunately, time restraints did not allow this. It now remains a firm target in any future work that will be conducted on this library.

6 Relevance to Environmental Protection Agency Ireland Policy and Future Work

It is the opinion of the authors of this report that this work is relevant to EPA Ireland policy in a number of areas, namely:

- Monitoring, analysing and reporting on the environment;
- Strategic environmental assessment;
- Environmental research and development; and
- Waste management.

The authors have studied and reported on the effects of a major environmental contaminant on an important soil species of nematode. The molecular and biological changes wrought by chromium VI in the nematode, and described here, are a substantial step forward in developing bioindicators and biomarkers to detect chromium in the environment, and which the authors believe will serve as a platform for the transfer of the technology to other test species for similar studies. Such a chain of developments will provide a series of easily performed tests (albeit, in skilled hands) that can inform on pollution events, assess their effects on flora and fauna, and monitor the progress of clean-up initiatives.

The research carried out during the course of this project can benefit from further work:

- Investigation into the effects of chromium VI on the offspring of *S. feltiae* parents that were exposed to chromium VI. These investigations would focus on both in vivo exposure and IJ-in-soil exposures. Such a study would help determine the long-term population effects of chromium VI on the fitness of the nematode.
- Molecular approaches, performed on exposed populations in tandem with the above investigations would encourage the development of determinable molecular characteristics that will be employed in large-scale molecular studies of nematodes in contaminated soils.
- Further sequencing and identification of the cDNA fragment inserts in the plasmids in the remaining subtracted library colonies may uncover yet more genes regulated by chromium VI. These genes, and the genes identified and described in this report, can be fully characterised, and investigations can be conducted to identify and characterise the unknown gene groups. These genes can then be developed as molecular indicators for the effects of chromium VI on nematodes in Ireland.
- It would be a valuable study to perform further trials to determine the actual chromium VI concentrations that induced molecular change. Here, 400-ppm chromium VI was used to induce the change required to allow subtracted cDNA library development, but this concentration was employed to ensure that such a change would occur. The results of such a study would enhance any future publication and inform on the construction of future experiments. Additionally, this research would benefit from work that investigates the lability of chromium VI in soils and in biological systems (i.e. insect cadavers). This work would augment understanding of the effects of chromium VI on ecosystems.
- These molecular indicators can then be applied to test nematodes living in contaminated environments in Ireland.

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Acronyms and Abbreviations

AAS	Atomic absorption spectroscopy
ANOVA	Analysis of variance
BVD	Bovine viral diarrhoea
cDNA	Complementary DNA
Cr VI	Chromium VI
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
EPN	Entomopathogenic nematode
GTC	Guanidinium thiocyanate
HCl	Hydrogen chloride
HSD	Honestly significant difference (Tukey's Test)
IJ	Infective juvenile
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB (broth/agar)	Lysogeny broth
MI	Maturity index
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
NaCl	Sodium chloride
NADH	Hydrogenated nicotinamide adenine dinucleotide
NaH_2PO_4	Monosodium phosphate
Na_2HPO_4	Disodium phosphate
$NaPO_4$	Sodium phosphate
PCR	Polymerase chain reaction
P + O ratio	Predator and omnivore ratio
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA

RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDS	Sodium lauryl sulfate
SI	Structure index
SPSS	Statistical Package for the Social Sciences (latterly known as *PASW (Predictive Analytics SoftWare* – 2009))
TE	Tris-EDTA
TR	Thioredoxin reductase
TRX	Redox protein thioredoxin
VIF	Variance inflation factor
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil agus Rialtais Áitiúil a dhéanann urraíocht uirthi.

ÁR bhFREAGRACHTAÍ

CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail.
- Scardadh dramhuisce

FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain.
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce.
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

NDP
National Development Plan 2007 - 2013

Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.

epa
Environmental Protection Agency
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