Soil contamination in urban Tyneside: A chemical and biological risk assessment

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DECLARATION

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where reference has been made to published literature. This work has not been accepted in any substance for any previous degree.

Zoe Alexandra Frances Keatinge

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RELATED PRESENTATIONS & PUBLICATIONS

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Soil contamination in urban Tyneside: A chemical and biological risk assessment

ABSTRACT

The extent of soil contamination with organic and inorganic pollutants in most urban areas in the UK is largely unknown but due to past and present industrial activity it is likely that pollutant levels are high. Such contamination could have a serious impact on human health. Therefore this thesis set out to examine the extent of soil contamination within Newcastle upon Tyne with a focus on the contribution of an incinerator (Byker) to contamination levels of urban soils. The Byker incinerator is situated in central Newcastle and has been the subject of much media controversy due to the disposal of incinerator ash on local allotments. The current work extended past investigations to see if the incinerator had contributed to general urban soil pollution (heavy metals and dioxins) by aerial deposition and allowed a useful investigation into levels of urban soil contamination in Newcastle. In addition to examining metal and dioxin levels the bioaccessibility (human and bacterial) of pollutants in selected soil samples was estimated and an attempt to develop a human cell based soil toxicity assay made. These measurements permit a preliminary assessment of risk to human health from soil contamination.

A total of 163 soil samples were collected based on predicted aerial deposition from the Byker incinerator and analysed for dioxins and heavy metals (As, Cd, Cr, Cu, Hg, Ni, Pb & Zn) content (mg pollutant /kg soil). A high proportion of samples (90/163) had contamination levels above soil guideline values (as proscribed in current UK regulations; CLEA). The highest dioxin levels were South West of the incinerator (1911 ng I-TEQ/kg) and the congener profiles coupled with a detailed historical survey demonstrated that the source of contamination was likely to be an old alkali works and not the incinerator. Overall it was found that the incinerator did not contribute significantly to dioxins found in the urban soils except those in the incinerator plant grounds. Soil metal contamination levels varied but were not related to incinerator deposition. Many samples contained levels of heavy metals well over soil guideline values with the highest values being found for Cu (12,108 mg/kg), Pb (4,134 mg/kg) and Zn (4,625 mg/kg)

To determine the potential human health risk associated with heavy metal contaminated soils, selected samples (16) were subjected to two *in vitro* digestion techniques which simulate the bioaccessibility of metals (Cu, Ni, Pb & Zn) to humans in case of soil ingestion. Only three samples had high levels of metal availability (over SGV's) indicating that these soils should be subjected to further risk assessment. All other samples tested had low metal availability most likely due to a combination of metal speciation, and complexation to soil particles over time.

An attempt was made to develop a human cell based system to determine the toxicity of contaminated soil. Using an *in vitro* system, human liver cells (HepG2's) were exposed to extracts from soils; cytotoxic effects (membrane integrity, metabolic capability and oxidative stress status) and genotoxicity potential (DNA damage) of Cu and Zn were first investigated in order to standardise the biological assays used. Between 0.1 and 10 mg/L Cu caused DNA damage and higher concentrations caused

cytotoxicity. Zn was also proven to cause genotoxic effects from 0.1mg/L. From 10 mg/L cytotoxic responses occurred and DNA damage could be attributed to cell death. Due to difficulties in sterilising soil extracts and physical damage caused to HepG2 cells by the abrasive nature of soil, it was not possible to elucidate whether metal contaminated soil extracts were capable of causing a cytotoxic or genotoxic response in human liver cells.

Finally, the same soil samples were then subjected to a bacterial (lux) biosensor technique to examine soil toxicity. Interestingly, despite the high levels of contamination found, none of the soil samples were found to be toxic to the two *Pseudomonas* strains used which again indicates a low level of ecosystem risk and suggests that most of the contaminants present are either in a form that is unavailable to living microbes or are complexed to soil particles.

In summary, this research has shown that the high level of soil contamination of urban areas in Newcastle is due to past industrial activity and a similar situation is likely in most other urban areas of the UK and internationally. The high cost of remediation means that if contaminated sites are shown to be a potential risk then bioaccessibility of contaminants should be examined in order to provide a more realistic assessment of the need for remediation. This work demonstrates that only a small proportion of urban contaminated sites are likely to require remediation based on bioaccessibility determination measurements.

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Abbreviations

AAS	Atomic absorption spectrophotometry
BGS	British Geological Survey
BSA	Bovine serum albumin
CLEA	Contaminated land exposure assessment
DEFRA	Department for Environment, Food and Rural Affairs
dH_2O	Distilled water
DMEM	Dubelccos Minimum Essential Medium
DMSO	Dimethyl sulphoxide
EA	Environment Agency (England and Wales)
ECACC	European Collection of Cell Cultures
EDTA	Diaminoethanetetra-acetic acid
FCS	Foetal Calf Serum
GCMS	Gas Chromatography Mass Spectrometry
GFP	Green fluorescent protein
GSH	Glutathione
HPLC	High pressure liquid chromatography
HR-GC/MS	High resolution gas chromatography and high resolution mass spectrometry
ICP-AES	Inductively Coupled Plasma – Atomic Emission Spectrometry
ICRCL	Interdepartmental Committee on the Redevelopment of Contaminated Land
ISO	International Standards Organisation
I-TEF	International Toxicity Equivalency Factors
I-TEQ	International Toxicity Equivalents; summary measure of toxic PCDD/F
LB	Luria-Bertani medium
LMPA	Low melting point agarose
MES	0.1 M 4-Morpholinoethanesulphonic acid hydrate
OD_{600}	Optical density at 600 nm
OECD	Organisation for Economic Co-operation and Development
OTM	Olive Tail Moment
PAH's	Polycyclic Aromatic Hydrocarbons
PBET	Physiologically Based Extraction Test
PBS	Phosphate Buffered Saline

PCB's	Polychlorinated Biphenyls
PCDD/F	Polychlorinated Dibenzodioxin/Polychlorinated Dibenzofuran
RLU	Relative light units
SGV's	Soil Guideline Values
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDI	Tolerable Daily Intake
USEPA	US Environmental Protection Agency
WHO	World Health Organisation

Chapter 1 - Introduction

1.1 Contaminated land

The soil environment is a sink for many chemicals transported in the atmosphere and given the long industrial legacy in the UK, is it probable that a large number of sites may be contaminated with levels of pollutants above natural background concentrations. Contaminated land may pose a threat to human health and the environment. Toxic effects may be acute (e.g. poisoning) or chronic (long term damage e.g. cancers or reproductive problems). The scale of the problem in the UK is such that the Environment Agency has estimated that over 20,000 sites or 300,000 hectares may be affected (Environment Agency, 2004a).The European Environment agency has estimated 1.5 million sites in Europe may be contaminated although only 300,000 sites have been assessed and deemed contaminated so far (European Environment Agency, 2004). Similar contamination problems exist in other countries throughout the world, for example, in the USA (~25,000 contaminated sites identified). In many other countries records of contaminated land are either sparse or non-existent; therefore the general scale of the problem is unknown.

In the UK, contaminated land is defined as:-

"Land which appears to the local authority in whose area it is situated to be in such a condition, by reason of substances in, on or under the land, that –

a) significant harm is being caused or there is a significant possibility of such harm being caused; or

b) pollution of controlled waters is being, or likely to be, caused."

This is an explicit statutory definition that is contained within the DETR Circular 02/2000, The Contaminated Land (England) Regulations 2000 (S.I.2000/227) and Section 57 of the Environment Act 1995 which was inserted into the Environmental Protection Act 1990 as Part IIA (www.defra.gov.uk).

Compounds which cause contamination and are likely to be present in soils include heavy metals, polyaromatic hydrocarbons (PAH's), pesticides and chlorinated solvents (Loomis & Hayes 1996). For purposes of analysis the two major subgroups of contaminants are metals and organics. The division into these groups is slightly misleading as often sites are contaminated with complex and heterogeneous mixtures of chemicals.

Urban soil contamination

Due to the rise in population (in the UK and worldwide) and the realisation that land is a finite resource, there is increased pressure to reclaim unused, "brownfield" sites; for example, in the UK, there is a current government target to ensure that 60% of new housing is to be built on brownfield sites in order to preserve the greenbelt. Unfortunately there is a lack of detailed knowledge about the extent of contamination that currently exists on these sites and throughout urban areas. There is a need to assess the extent of urban contamination to ensure that the contamination does not negatively impact on human health and the environment.

For example, a recent British Geological Survey (BGS) survey of Arsenic in urban soils in Sheffield, UK (Cave *et al.*, 2002) found that over 60% of samples were contaminated to levels above soil guideline values (SGV's) set by CLEA (Environment Agency, 2002b) and it is likely that similar results will be found for all UK cities with an industrial history including Newcastle upon Tyne and the greater Tyneside area.

Common "point sources" (as opposed to diffuse sources such as vehicle emissions) of soil contamination in urban areas include transport-related hubs (docks, railways, coach works, engineering works etc) industrial and chemical manufacturers and processors, and waste disposal sites including landfill sites and incinerators. A review of industrial uses of land (in the Tyneside area) that have been associated with contamination is included in Vizard *et al.*, (2003).

Common pollutants from incinerators

Priority pollutants produced by incinerators include heavy metals and dioxins. Dioxins can be formed from the incomplete combustion of (chlorinated) mixed wastes. Heavy metals are present in many of the raw components of domestic waste. For example, batteries (mercury, nickel, cadmium), lead based paints, copper pans and pipes are all common municipal waste items. A range of factors (e.g. temperature, component mixture, aeration) determine levels of heavy metals given off in emissions from incinerators, although with improved air abatement technologies, levels have dropped (Allsopp *et al.*, 2001) dramatically over the past decade. Despite this there is still concern that previous and current emissions have added to background levels of contamination in soils.

Urban soil contamination in Tyneside

Newcastle upon Tyne (and the greater Tyneside area) is an area (of the UK) that has supported high levels of industrialisation for over 300 years and given that there is a lack of detailed information outlining the scope of urban soil contamination it is pertinent to ask whether Newcastle's industrial heritage may pose a risk to public health.

This work specifically looks at soils surrounding an incinerator site in Byker, Newcastle upon Tyne. Triggered by an application to increase capacity and extend the height of one of the incinerator stacks (Environment Agency Public Register 1998), it was discovered that ~2000 tonnes of potentially toxic fly ash from the plant had been used around various sites in Newcastle (mainly on pathways in allotment sites, 44 locations in total). A local campaign group, BAN waste (www.banwaste.org.uk), concerned with the health impact of the incinerator and ash distribution pressured Newcastle Council to investigate this. An initial investigation into the levels of dioxins and heavy metals (common incinerator emissions and ash contaminants) in affected footpaths and surrounding soils (Pless-Mulloli *et al.*, 2000) recommended further work to assess and geographically examine whether depositions from the incinerator stack and fugitive emissions had any impact on levels of soil contamination (with dioxins and heavy metals) in the surrounding area.

The common types of urban soil pollutants are discussed in the following section.

1.2 Dioxins – properties and toxic effects

Polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/F's), more generally referred to as "dioxins", are 210 structurally related compounds (or congeners -75 dioxins and 135 furans) which exist in complex mixtures and are regarded as persistent organic pollutants. Examples of dioxin and furan structure are shown in Fig 1.1.

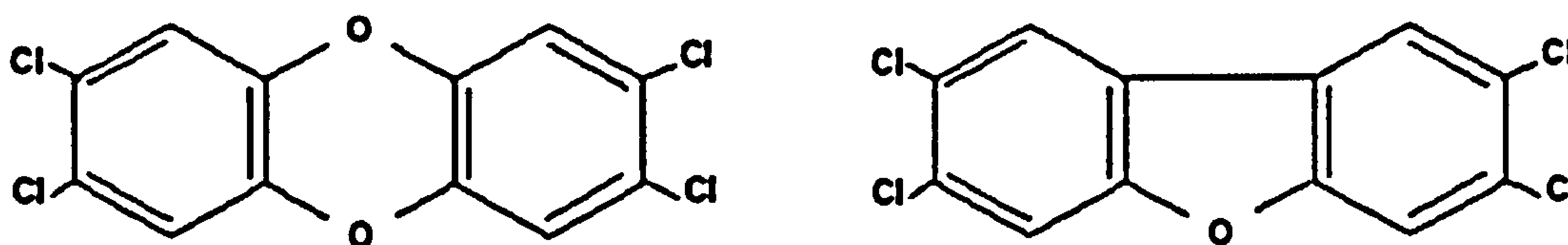


Figure 1.1 An example of a dioxin structure (2,3,7,8-tetrachlorodibenzo-p-dioxin – TCDD, left) and a furan structure (2,3,7,8-tetrachlorodibenzo-p-furan –TCDF, right).

Dioxins are formed either as undesired by-products of chemical processes (e.g. pesticide manufacture) or by incomplete combustion of chlorinated wastes (e.g. burning mixed wastes); as such, a substantial amount of research examining pollution from incinerators (e.g. Nouwen *et al.*, 2001) has focused on dioxins. Regulatory and public perception of the health effects of dioxins has been coloured by several major public health incidents involving dioxin toxicity. These include:-

Agent Orange herbicide, used as a defoliant in the USA:Vietnam conflict, was found to be contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and is believed to have contributed to a range of illnesses including chemical acne, Hodgkin's lymphoma and soft-tissue sarcomas in war veterans (www.agent-orange-lawsuit.com).

In 1976, an explosion in a chemical plant in Seveso, Italy led to a cloud containing TCDD (also now known as the "Seveso dioxin") being released over a densely populated $6km^2$ area. The definitive health effect reported at the time was chloracne (>200 cases)(Bertazzi *et al.*, 1998). Long term monitoring found increased diabetes, chronic circulatory and respiratory diseases, cancers of gastrointestinal sites and of the

lymphatic and haematopoietic tissues (Bertazzi *et al.*, 1998; Bertazzi *et al.*, 2001) although results could not be viewed as conclusive.

In late 2004 the Ukrainian opposition leader, Viktor Yushchenko, was poisoned by pure TCDD, which resulted in highly disfiguring facial lesions and cysts (chloracne), acute pancreatitis, liver problems and other ailments (see Fig 1.2). His blood levels of pure TCDD dioxin were found to be ~100,000 pg/g blood fat, six thousand higher than normal (http://news.bbc.co.uk/2/hi/health/4041321.stm)

Figure 1.2 Contrasting pictures of Viktor Yushchenko before and after dioxin poisoning (Associated Press, 2004)

The mechanism of action of dioxins in humans is binding to the Ah (aryl hydrocarbon) receptor upon which the receptor then dissociates from its protein complex and influences transcription by interacting with various cell signalling pathways involved in development and homeostasis (Hahn, 1998).

A central concern with dioxins is persistence, both in the environment and in the human body; due to the lipophilic nature of these organic molecules, bioaccumulation occurs up the food chain. Most dioxin congeners are not regarded as a threat to human health although 17 are thought to be toxic and may pose a range of risks. These problems include carcinogenicity, immune system problems, reproductive problems (male and female), developmental impacts, hormonal changes, organ damage and many more (WHO, 1998). TCDD is the most toxic and is regarded as a class 1 carcinogen (IARC, 1997). As dioxins occur as complex mixtures, with variable levels of toxicity, an internationally recognized system was established (NATO/CCMS, 1988a; NATO/CCMS, 1988b) in order for data to be reported in a fashion that was compatible with other studies. The International Toxicity Equivalency Factors (I-TEF) are used in order to establish the potential for toxicity, and are translated into

International Toxicity Equivalence Factors (I-TEQ) (see Table 1.1). For soil, results are translated in ng I-TEQ/ kg soil.

A slightly different system is promoted by the World Health Organisation (WHO) (WHO, 1998); this system also includes PCB's with dioxin-like action. The WHO has established a Tolerable Daily Intake (TDI) for humans; based on the lowest doses shown to cause adverse effects in experimental animals (a safety factor of 10 has also been applied). The level is currently 1 to 4 pg I-TEQ / kg body weight / day, averaged over a life time, with the eventual goal of reducing human intake below 1 pg I-TEQ / kg body weight / day (WHO, 1998).

Table 1.1 International toxicity equivalent factors (I-TEF)

(in accordance with NATO-CCMS)

Congener	I-TEF
2,3,7,8 TCDD	1
1,2,3,7,8 PeCDD	0.5
1,2,3,4,7,8 HxCDD	0.1
1,2,3,6,7,8 HxCDD	0.1
1,2,3,7,8,9 HxCDD	0.1
1,2,3,4,6,7,8 HpCDD	0.01
OCDD	0.001
2,3,7,8 TCDF	0.1
1,2,3,7,8 PeCDF	0.05
2,3,4,7,8 PeCDF	0.5
1,2,3,4,7,8 HxCDF	0.1
1,2,3,6,7,8 HxCDF	0.1
1,2,3,7,8,9 HxCDF	0.1
2,3,4,6,7,8 HxCDF	0.1
1,2,3,4,6,7,8 HpCDF	0.01
1,2,3,4,7,8,9 HpCDF	0.01
OCDF	0.001

All dioxin congeners are likely to be produced from all waste incinerators (independent of type or waste composition, see DCDEP, 1989) although the make-up will be affected by variations in the waste burnt, temperature used, rates of feed and various other factors. It is possible to (tentatively) identify the source of some dioxin contamination by using homologue group "fingerprints" (a homologue group is the sum of the compounds with the same number of Cl atoms) as some industrial processes produce distinct patterns ref (e.g. Creaser *et al.*, 1990; Zook & Rappe, 1994 and see Figure 2.17).

1.3 Arsenic and heavy metals – properties and toxic effects

As described in 1.1, heavy metals are likely to be found as the components of raw domestic waste and consequentially in emissions from incinerators and therefore may have added to contamination of urban soils.

Arsenic and heavy metals (density > $6g/cm^3$ and atomic number > 20) are naturally occurring elements, found throughout the earth in rocks and soil, but levels tend to be elevated in soils by pollution and contamination. Common sources of metal pollution are mining, metal manufacturing and refining industries (e.g. smelters), chemical works, timber works, engineering works (e.g. docks and railway yards), munitions and other warfare related activities and waste disposal (Alloway, 2001).

Sometimes known as trace elements, some heavy metals are essential micronutrients (e.g. Co, Cr, Cu, Mn, Mo, Ni, Se and Zn) for growth processes but at excessive concentrations, metals are toxic to living organisms, including humans. Heavy metals have been shown to cause harm to humans both acutely and chronically with toxic end points that include damage to neurological, haematopoietic, renal and hepatic function and carcinogenesis. Much of the research into the effects of heavy metals on human health has focussed on arsenic, lead, cadmium and mercury (all non-essential metals).

Factors that influence the toxicity of metals to humans include interactions with other (essential) metals, formation of metal protein complexes, the age and stage of

development of the person and their immune status and the chemical form or speciation of the metal (Klaassen, 2001).

The most thorough and up to date information on the toxicology of heavy metals (and many other substances) is available from the US Agency for Toxic Substances and Disease Registry (www.atsdr.cdc.gov).

Arsenic

The principal use of arsenic is as a wood preserving agent (arsenic trioxide) but it is also found in agricultural chemicals (insecticides, herbicides, etc) and used in the electronics industry. The oxidation state of arsenic is important; it occurs naturally in trivalent and pentavalent states but the trivalent form is principally toxic (Bissen & Frimmel, 2003).

Ingestion of a large dose (10-180 mg) can be fatal. Acute illness symptoms that can be caused include fever, anorexia, hepatomegaly, melanosis and cardiac arrhythmia which all lead to cardiovascular failure. Peripheral nervous system sensory loss can also occur but is reversible (Klaassen, 2001).

Chronic exposure effects include peripheral and central nervous system neurotoxicity and liver injury leading to jaundice and cirrhosis. There is still some debate as to whether As may be a minor essential element as laboratory animals are less sensitive to its effects than humans; sufficient evidence is available to show that As causes cancers in humans but only limited evidence is available to show it causes cancer in animals (Mandal & Suzuki, 2002). Carcinogenic effects seen include skin cancers on areas not usually exposed to sunlight and when ingested or inhaled, cancers of the lung, liver, kidneys, bladder and lymphomas and leukaemia's (www.cie.iarc.fr).

Cadmium

Cadmium is a toxicant of increasing importance; it was only discovered as an element in 1817 and used sparingly until 50 years ago. Common uses include electroplating and galvanizing, colour pigmentation, battery material (Ni-Cd batteries) and it is a by-

product of Zn and Pb mining and smelting. It is also present in cigarette smoke and as a phosphate fertiliser contaminant in (Klaassen, 2001).

Acute toxic effects after ingestion can include abdominal pain, nausea and vomiting and after inhalation may cause acute chemical pneumonitis and pulmonary oedema. Chronic exposure can lead to chronic obstructive pulmonary disease, emphysema, chronic renal tubular disease and possibly cardiovascular and skeletal system problems (Jarup, 2002). Cadmium is also known to cause cancers in both animals and humans. Specific sites of carcinogenesis include the lungs and prostate (Satoh *et al.*, 2002).

Chromium

Chromium is a naturally abundant element and an essential trace element (micronutrient) e.g. it is an insulin co-factor. Industrially it is used in stainless steel production, tanneries, cement factories and textile plants (Klaassen, 2001). It exists with a range of oxidation states but only the trivalent and hexavalent states are biologically significant; in biological material all Cr is trivalent with the hexavalent molecules being converted to a trivalent state intracellularly. Toxicity may occur by accidental exposure, overdose or therapeutic uses and the main effect seen is kidney damage. Hexavalent Cr overexposure may have a corrosive effect including ulceration and perforation of the nasal septum and skin ulcers (Zayed & Terry, 2003). Occupational exposures have been associated with cancers of the respiratory tract (the conversion from hexa- to tri-valent and generation of radical intermediates is thought to be the mechanism involved, Dayan & Paine, 2001).

Copper

Cu is a widely distributed and essential (micronutrient) element. Illness and toxic effects are seen more commonly from deficiency than over exposure (e.g. anaemia from defective haemoglobin synthesis). Cu is required by oxidative enzymes (catalase, peroxidase, cytochrome oxides etc) with body stores regulating absorption and bile as the major excretion pathway. Storage is mainly in the liver and bone (serum levels 120-145 μg/L) bound to metallothioneins (Klaassen, 2001).

Acute poisoning from excessive oral ingestion causes vomiting, hypotension, coma, jaundice and hepatic necrosis (Cai *et al.*, 2005). Regulation levels for Cu in US drinking water are 1.3 mg/L (www.epa.gov/safewater/regs).

Chronic exposure can lead to Indian childhood cirrhosis (water ingestion related) and chronic cholestatic conditions. There are two inherited disorders of Cu metabolism, Wilson's disease and Menke's disease. In Wilson's disease, excessive accumulation of Cu in the liver, brain, kidneys and cornea with high urinary excretion levels, lead to clinical abnormalities; it can be treated by chelation with penicillamine (Ferenci, 2004). Menke's disease (also know as kinky hair syndrome) is a sex linked disease characterised by peculiar hair, failure to thrive, severe mental retardation, neurological impairment and death before 3 yrs. Low levels of Cu are found in the liver and brain, with high concentrations in other tissues (Daniel *et al.*, 2004).

Mercury

Hg exists in organic and inorganic forms with speciation leading to different effects. Use of Hg in most industrial processes (e.g. mining and smelting) has been curtailed. Most Hg is produced from natural degassing of the earths crust; most exposure is by inhalation. Hg vapour diffuses into the blood steam and has an affinity for red blood cells and the central nervous system (Klaassen, 2001). Other common routes of exposure are occupational and via eating contaminated seafood (Fuentes & Gil, 2003). Oral ingestion of metallic Hg poses no risk as none is absorbed, ~ 7% of inorganic Hg is absorbed when ingested (biological half time 40 days) whereas organic methyl mercury is absorbed at rates of 90-95% (70 day biological half time); it concentrates in the kidneys and brain and passes to the foetus through the placenta on a positive gradient. Acute exposure by inhalation of mercuric vapours can lead to corrosive bronchitis and central nervous system tremor and excitability. Acute effects of oral ingestion of mercuric salts can include corrosive ulceration, bleeding, necrosis of the gastrointestinal tract, shock, circulatory collapse and finally renal failure. Genotoxic effects (chromosomal aberration) have been demonstrated in mercury exposed populations but evidence of carcinogenesis in humans is inadequate (Tchounwou *et al.*, 2003).

Nickel

There is growing evidence that Ni may be an essential trace metal in plants. In humans it is absorbed sparsely by ingestion and excretion is in the urine 4-5 days later. Induction of metallothioneins in the liver and kidney is only slight. The most common toxic effect of Ni is allergic contact dermatitis (4-9% of people effected). Excessive exposure to nickel carbonyl has been reported to cause headaches, nausea, vomiting respiratory failure and cerebral oedema leading to death (Denkhaus & Salnikow, 2002). Chronic exposure can lead to respiratory tract (lung and nasal), liver, kidney and brain cancers.

Lead

Lead is ubiquitous in the both the inert and biological environment and one of the most infamous of pollutants. At high exposures (doses) Pb is toxic to almost everything. Food is the principal exposure route (current dietary intake <20 μg/day from 500 μg/day in the 1940's) with environmental exposure (paint, drinking water, lead-glazed pottery) usually producing the excess and toxic exposures. Removal of Pb from petrol has reduced airborne levels significantly. In adults 5-15% is absorbed during oral ingestion with <5% retained; levels of absorption are greater in children (~30-40%). Pb in blood resides in the red blood cells with the main body burden (75-90%) in the bones (biological half time > 20 years) (Klaassen, 2001).

The central nervous system and grey matter is the most significant target in children with significant developmental effects possible. In adults, anaemia, hypertension, neuropathy and nephropathy, gastrointestinal and reproductive system effects are most likely. The carcogenicity of Pb is debated, with respiratory and digestive systems cancers found to be slightly increased (Papanikolaou *et al.*, 2005).

Zinc

Zinc is a nutritionally essential (micronutrient) element that is ubiquitous in the environment. Excessive exposure is very rare and it does not tend to accumulate in the body with repeated exposure as liver levels and absorption are homeostatically regulated. Zinc is bound to metallothionein synthesis, is found in greatest concentrations in the prostate and is excreted in urine. It is required by >200 enzymes as a co-factor and deficiency, rather than over-exposure, is a more common problem.

Deficiency of Zn can cause dermatitis, developmental problems, poor wound healing and can exacerbate impaired copper nutrition. Interactions of Zn with Cd and Pb may modify the toxicity of these metals (Cai *et al.*, 2005). Toxicity from excessive ingestion has been reported and effects included gastrointestinal distress and diarrhoea. Excessive industrial exposure to metal fumes (mainly zinc oxide) can cause fever, chills, sweating and weakness. Zinc is not thought to be carcinogenic (Klaassen, 2001).

1.4 Definitions, assessment and regulation of contaminated land

As outlined in sections 1.2 and 1.3 it is clear that potential contaminants are toxic and if present will cause harm to both humans and ecosystems. This risk of harm has led to development of regulations in different countries in an effort to protect vulnerable receptors. Guidelines have been set that outline levels of contaminants, above which, soils may be considered to be a risk. Different countries have generic assessment criteria - set intervention values for specific contaminants. Variation in the intervention values given by different countries occur mainly from "uncertainty surrounding the toxicological database and differences in policy on exposure and averaging periods" (Environment Agency, 2002b)

Site risk assessment (UK based approach) must first consider whether a) contamination exists that is b) able to follow a pathway to c) a receptor (humans/flora and fauna/water resources/buildings, services and structures) which may be adversely affected. The focus of the majority of environmental legislation is the protection of human health.

Interdepartmental Committee on the Redevelopment of Contaminated Land (ICRCL) – England and Wales, 1980's-2002

Levels of contaminants in soil were first regulated in the UK in the early 1980's by the ICRCL (ICRCL, 1987). Generic criteria (threshold trigger value and action trigger value) were derived for specific chemicals (As, Cd, Cr, Pb, Hg, Se, B, Cu, Ni, Zn, cyanides, sulphates, PAHs and phenols) above which levels, a site was deemed to be

contaminated. This approach, whilst easy to understand was not thought to be sufficiently scientifically robust as it took no account of the variability of soils, chemical states of compounds and had set levels several orders of magnitude below those thought to increase risk of harm. The limitations of this regime were recognised and have since been superseded by the Contaminated Land Exposure Assessment (CLEA) model.

Contaminated Land Exposure Assessment (CLEA) - England and Wales, 2002

Contaminated land regulations in England and Wales are set and administered by the Environment Agency. The primary aim of these regulations is to protect human health from chronic risks in relation to land use, with the environment also being considered. Human health risk assessment is managed using the CLEA regime (Environment Agency, 2004b). Soil guideline or intervention values (SGV's – see table 1.2) have been derived, above which a site may be presumed to pose an unacceptable risk to users (i.e. the regime only looks at human health) prompting further investigation and possible remediative action.

Table 1.2 Summary of Environment Agency and DEFRA Contaminated Land Exposure Assessment Model Soil Guideline Values

SGV number	Element	Residential with plant uptake and Allotments	Residential without plant uptake	Commercial and industrial
		SGV (mg/kg)	SGV (mg/kg)	SGV (mg/kg)
SGV1	Arsenic	20	20	500
SGV3	Cadmium	pH6- 1, pH7- 2, pH8- 8	30	1400
SGV4	Chromium	130	200	5000
SGV5	Inorganic mercury	8	15	480
SGV7	Nickel	50	75	5000
SGV9	Selenium	35	260	8000
SGV10	Lead	450	450	750

Values given in mg/kg dry weight soil. (Environment Agency, 2002b)

CLEA is a much more sophisticated model than ICRCL and takes into account exposure pathways, end use of the land, and starts to consider the complexity of the soil environment (e.g. pH) including the behaviour of contaminants.

Table 1.3 shows an outline of the key CLEA reports.

Table 1.3 Summary and outline of key CLEA reports

CLR7 – ***Assessment of Risks to Human Health from Land Contamination: An Overview of the Development of Soil Guideline Values and Related Research***	This report is an introduction to the other reports in the series; the legal framework is set out including the statutory definition of contaminated land (under Part IIA of the Environmental Protection Act 1990).
CLR8 – ***Priority Contaminants Report***	Contaminants (and families of contaminants) which are a priority are set out in this report. Those included are contaminants that are found in sufficient quantities to cause harm or pose a risk to humans, buildings, water or ecosystems and are usually found on sites affected by industry or waste management activity
CLR9 – ***Contaminants in Soils: Collation of Toxicological Data and Intake Values for Humans***	The approach to selecting TDI's and Index doses for contaminants is set out in this report.
CLR9 TOX 1-10	TDI's or index doses for the first 10 SGV's (including As, Benzo(a)pyrene, Cd, Cr, cyanide, Pb, phenol, Ni, Hg inorganic compounds and Se) are detailed in these reports
CLR10 – ***Contaminated Land Exposure Assessment Model (CLEA): Technical basis and Algorithms***	The conceptual exposure models for standard land-uses that are used to derive SGV's and technical algorithms for modelling exposure are contained in this report.
CLR10 – SGV 1-10	The derivation of the first 10 SGV's is set out in these reports. SGV's determined include As, Benzo(a)pyrene, Cd, Cr, cyanide, Pb, phenol, Ni, Hg (inorganic compounds) and Se.

(adapted from Environment Agency, 2002c).

The regime is based on the SOURCE→→PATHWAY→→RECEPTOR model i.e. if a contaminant exists in soil and there is a pathway to a receptor (child, ecosystem, waterway etc) then further risk assessment should go ahead.

Particularly toxic and prevalent (priority) contaminants were identified (CLR8) and tolerable daily intakes (TDI's) and index doses investigated (CLR9 and TOX1-10) in order to derive SGV's (CLR10 SGV1-10). A detailed account of the derivation of SGV's is not given here, merely an overview. Exposure pathway (shown in table 1.4), chemical properties of a contaminant, health effects of a contaminant and eventual land use are all important in any conceptual exposure model.

Table 1.4 Exposure pathways to contaminated soil

Exposure pathway
Outdoor ingestion of soil
Indoor ingestion of dust
Consumption of home-grown vegetables
Ingestion of soil attached to vegetables
Skin contact with outdoor soil
Skin contact with indoor dust
Outdoor inhalation of fugitive dust
Indoor inhalation of dust
Outdoor inhalation of soil vapour
Indoor inhalation of soil vapour

(adapted from Environment Agency, 2002c)

Overall, CLEA is a pragmatic set of regulations that acknowledges that it is built on incomplete information and further research is needed. A balance is established between redevelopment and cost and a generic approach to all sites in the UK is not used. Sites are assessed according to the use the land will be put to e.g. houses with gardens or a capped car park, and worst case scenario assessment is not encouraged. The guidance was produced so a transparent and consistent approach (nationally) is possible and CLEA can act as an aid to planning regulations to ensure health standards by trying to assess long term chronic risks.

Other contaminated land guidance

A range of other contaminated land regimes exist in the UK and internationally including SNIFFER (SNIFFER, 2000), Dutch government regulations (VROM, 2000) and the USEPA superfund project (USEPA, 1996). The basic principle of protecting human health from unnecessary exposure to toxic compounds is the main premise of all these schemes but huge variation exists in the detail. The fundamental differences include the range of contaminants examined, the receptor types prioritised, the land use and exposure scenarios and whether the source-pathway-receptor approach is taken.

Soil guideline values for dioxins, arsenic and heavy metals

Guideline values for levels of dioxins in soil, above which further assessment or remediation should take place, have been set in several countries. A summary of current levels can be seen in Appendix 1, Table A1.1.There is currently no defined intervention or soil guideline value set for dioxins in the UK although as a "TOX report" (CLEA, TOX 12) that collated toxicological data on dioxins, furans and dioxin-like PCB's is available, a SGV is likely to follow in the near future.

Table 1.5 summarises the CLEA documents available that relate to As and heavy metals and also gives the location in Appendix A1 where summaries of international guidelines can be found.

Table 1.5 Summary of the CLEA documents

Metal	**CLEA documents**	**International guideline summary**
As	TOX1 and SGV1	Appendix 1, Table A1.2
Cd	TOX3 and SGV3	Appendix 1, Table A1.3
Cr	TOX4 and SGV4	Appendix 1, Table A1.4
Cu	None; ICRCL most recent	Appendix 1, Table A1.5
Hg	TOX7 and SGV5	Appendix 1, Table A1.6
Ni	TOX8 and SGV7	Appendix 1, Table A1.7
Pb	TOX6 and SGV10	Appendix 1, Table A1.8
Zn	None; ICRCL most recent	Appendix 1, Table A1.9

(relating to As and heavy metals and location of summary of international guidelines).

Ecological risk assessments

A need to assess contaminated land and its potential effects on ecological receptors has also been recognised. Assessment of soil contamination for the sake of environmental protection is currently in draft form in the UK. It will use an Ecological Risk Assessment (ERA) scheme to protect ecosystems (Environment Agency, 2003). This is also regulated by the Environment Agency. In a scheme similar to CLEA, specific numbers, soil screening values (SSV's), for priority contaminants will be given, above which, intervention will be required (see Table 1.6).

Table 1.6 Priority contaminants likely to be found at potentially contaminated sites in the UK.

Metals	Arsenic, Beryllium, Cadmium, Copper, Lead, Mercury, Nickel, Selenium, Zinc.
Organometals	Organolead compounds, Organotin compounds, e.g. tributyltin.
Inorganics	Cyanides
Aromatics	Total Petroleum Hydrocarbons, Benzene, Toluene, Ethylbenzene, Xylene(s), Phenol.
Polycyclic Aromatic Hydrocarbons (PAHs)	Benzo(a)pyrene, Anthracene, Naphthalene, 1,2,4-trichlorobenzene
Chlorinated Hydrocarbons	Tetrachlorobenzene, Pentachlorobenzene, 1,2-Dichloroethane, 1,1,1,-Trichloroethane, Tricholorethene, Tetrachloroethene, Pentachlorophenol, Chlorotoluenes, Vinyl chloride, Chloroform, Hexachlorobuta-1,3-diene, Polychlorinated biphenyls (total), Dioxins and furans.
Pesticides	Dieldrin, DDT (total), HCH (total).

Soil screening values will be developed for these contaminants (Environment Agency, 2003).

1.5 Limitations of the current regulatory approach to the assessment of contaminated land

Assessment of a potentially contaminated site is currently carried out by chemical analysis which determines "total" amounts of a contaminant in a soil. Advantages of this approach are that it tells you what contaminants you actually have in a site. Limitations of this approach include the fact that you can only find what you specifically look for (unknown or unexpected contaminants may be present), exquisite sensitivity is needed to look for low amounts of chemicals and finally chemical analysis can be very costly. Indeed, CLEA (and other contaminated land regimes) only identify a small set of priority contaminants, ones known to be very toxic or mutagenic (Environment Agency, 2003; Environment Agency, 2004b). These methods, although very convenient for regulation and analysis are unrealistic due to (historic) contamination being a highly complex "mixed bag" of compounds. Chemical analysis is unable to give any indication of potential synergistic/additive effects of contaminants or antagonistic effects (La Point & Waller, 2000). Contaminants may have the same or similar mode of action and as such magnify any toxic response or alternatively contaminants may compete for a pathway, reducing each others capacity for toxicity. For example, An *et al.*, (2004) investigated the toxicity of copper, cadmium and lead to plant growth and found additive, synergistic and antagonistic effects. This means that chemical analysis alone is not able to accurately predict the biological response to mixtures of contaminants in soil.

Another flaw to total chemical analysis is that it is unable to predict what percentage of any contaminant is biologically available (bioavailable) to cause harm. If a compound is not bioavailable it therefore cannot be toxic. Bioavailability therefore should be a major consideration when assessing potential soil toxicity. Bioavailability gives a more realistic view of the risk a site may pose to humans or the environment. Given the high predicted levels of contaminated land in the UK and internationally, the cost of remediating all such sites would be prohibitive. Clearly if bioavailability assays can indicate that contaminated soils that may present a risk, are in fact low risk, and do not need to be remediated then this will represent a significant cost saving. Another reason for examining bioavailability is for determining the potential success

of any (bio)remediation techniques used. The complexity of soil makes defining bioavailability an even more complex question.

1.6 Bioavailability: concepts and limitations

As with many scientific terms, usage and definitions of bioavailability can vary. The Environment Agency uses *bioavailable* as meaning "the fraction of the chemical that can be absorbed by the body, through the gastrointestinal system, the pulmonary system and the skin" and *bioaccessible* as meaning "the fraction of a substance that is available for absorption by an organism" (Environment Agency, 2004a). The US National Research Council report on Bioavailability of Contaminants in soils and sediments (Ehlers & Luthy, 2003; National Research Council, 2002) has no explicit definition of bioavailability. It defines *bioavailability processes* as the "individual physical, chemical, and biological interactions that determine the exposure of organisms to chemicals associated with soils and sediments". The major factor that these definitions do not include is the dynamic aspect of bioavailability over time. Schulin's summary of the 2003 Bioavailability Workshop (Schulin, 2003) discusses the problems of defining bioavailability. It talks about how contaminant uptake depends on delivery rate from soil to an organism and how definitions based on momentary concentrations are inaccurate compared to ones based on fluxes and rates of re-supply (also see Peijnenburg & Jager, 2003). It then goes on to say that for practical purposes the best approach is to describe bioavailability in terms of soluble or labile concentrations; that soil solutions concentrations of a contaminant are nearer to bioavailability than a total concentration in soil.

Factors affecting bioavailability of contaminants in soil:

A detailed discussion of bioavailability is outside the scope of this section; a brief overview is given below. There are several well documented factors which affect bioavailability including:

- The soil itself
- The specific contaminant and co-contaminants present
- The particular organism(s)/ecosystem at risk
- Residence time of a contaminant in soil

1. Soil: basic soil properties which need to be considered that may influence bioavailability are; pH (Impellitteri *et al.*, 2003; Lock & Janssen, 2003) redox potential (Rensing & Maier, 2003), ionic strength, organic matter (Pardue *et al.*, 1996), type of soil (Lock *et al.*, 2002), clay fraction (Babich, 1977), water content, oxygen content, temperature and soil residents (organisms, plant roots, invertebrates etc).

2. Contaminant physicochemical properties that may affect bioavailability include molecular structure, polarity, aqueous solubility, lipophilicity (the major factor affecting bioaccumulation in food chains and secondary poisoning) and volatility (Reid *et al.*, 2000), speciation of metals (Arnold *et al.*, 2003), mineral form (Davies *et al.*, 2003) mobility and persistence. For more detailed information on the bioavailability of organics see Stokes *et al.*, (2004); on pesticides see Gevao *et al.*, (2003) and on metals see Rensing & Maier, (2003). Co-contaminants may have an unpredictable effect and may act synergistically or competitively with each other.

3. The organism for which risk is being assessed, whether it be a microbe, invertebrate, plant, fish (sediment) or humans: different organisms all have completely different routes of exposure (for example microbes at soil pore microscopic level, humans at a macroscopic level. Different compounds will cause dissimilar toxic effects depending on species exposed (different reactions can and will be seen even at the individual level). For example Lock

et al., (2002) examined the toxicity of lindane to various soil invertebrates. They discovered that toxicity was species specific and not dependent (in this instance) on organic matter content of the soil. If organisms within the same feeding groups exhibit different sensitivities it may therefore be almost impossible to generalise or talk about the relevance of one organism to another.

4. Residence time of contaminants is one of the factors most talked about and difficult to define. Referred to as "ageing"; a time dependent interaction between the contaminant and the soil. Contaminants become sorbed to mineral and organic matter components of soil and trapped in micropores and become generally biologically inaccessible. The longer a contaminant is in contact with the soil, the more the soil and contaminant become associated, reducing bioavailability and consequent potential toxicity (Alexander, 2000; Hatzinger & Alexander, 1995).

Assessment of contaminant bioavailability in soil

The bioavailability of contaminants tends to be mimicked using extraction methods (chemical methods of determining bioavailability). It is important to consider and understand the advantages and limitations of these extraction methods as many of them are used as the first step in assessing the bioavailability of pollutants to actual organisms. The soluble or labile fraction from a soil has usually acted as a guideline for bioavailability. The US NRC report (Ehlers & Luthy, 2003; National Research Council, 2002) gives a thorough review of the physical, chemical and biological tools used to measure bioavailability. Categories include physical and chemical characterisation of the soil (referred to as the solid phase as the report also covers sediment analysis), extracts for inorganic contaminants and extracts for organic contaminants.

Extracts for inorganic contaminants tend to fall into three groups; conventional or single, sequential and passive. Conventional tests simulate a simple leaching procedure or phytoavailability in a single extraction using water, acid, chelating agents or salt solutions (Rauret, 1998). Sequential methods use similar agents to single extractions but are designed to differentiate between elements associated with different physico-chemical soil phases (for example Ure *et al.*, 1993). Passive techniques involve exchangeable resins or pore water measurements. Extracts for organic contaminants tend to use solvents; "total" concentration of organics involves use of an organic solvent and either heating or shaking (Stokes *et al.*, 2004).

An area, mentioned briefly in the NRC report, of bioavailability research that has received increasing attention is that of *in vitro* tests to mimic human intake for both organics and inorganics. Such tests appear to be an interface between purely chemical extraction assays and those which use living organisms.

Human exposure routes to contaminated soils are inhalation, ingestion and dermal contact. Ingestion of soils, or oral bioavailability, is considered the highest priority exposure route (Paustenbach, 2000). Due to the increasing recognition of the importance of oral bioavailability different groups in different countries in parallel have designed and developed their own version of an *in vitro* simulated gut extract, all based on the same principles but with significant variety in the experimental detail. Several of these are simultaneously assessed and reviewed in Oomen *et al.*, (2002). This topic is also expanded upon in the Environment Agency technical report of 2002 (Environment Agency, 2002a). The basics of the various techniques include processes that mimic the stomach (low pH and physical agitation) then the intestines (rise in pH and addition of digestive enzymes) all at physiological temperature, 37°C. The features of each method that may lead to differences in contaminant bioavailability include the pH of the "stomach", time taken at different pH's and whether food is added as well as soil. Several methods have been connected to *in vivo* studies and validated for certain contaminants with the fullest summary being given in Kelley *et al.*, (2002).

These different *in vitro* extraction methods appear to give very different measures of bioavailability (also see Harkey & Young, 2000) depending on the technique used and the contaminant being studied.

The advantages of chemical/*in-vitro* extraction techniques to evaluate bioavailability of contaminants in soil are reproducibility of conditions, speed of analysis and potentially greater relevance to biological systems than measurement of "total" amounts of contaminants. The major limitation of the majority of methods described is that most of the extracts produced still have to be chemically analysed and are not suitable to then go on and use with biological tools to determine biological availability of contaminants/actual toxicity of a soil. For example, acid extracts and low pH simulated gut extracts would denature/kill pH sensitive molecules/organisms; organic solvents alone tend to be toxic to biological systems and *in-vitro* intestinal enzymes will digest and break down biological matter. In addition the majority of extracts are designed to examine either availability of metals or organics, which again is an unrealistic picture of mixture contaminated soil. Extracts may over or under-estimate bioavailability to different species especially as different organisms are exposed to different soil fractions. Also as mentioned previously, different species have different sensitivities to metals/organics and what a chemical assay considers "available" may not affect an organism. Therefore the choice of extraction method used for bioassays is important and deserves critical attention.

1.7 Biological assays

Biological assays can be used to assess the toxicity of individual compounds (metals, pesticides, and potential pharmaceutical products) or the general response to complex mixtures, (for example, contaminated soil) and so are a useful tool in bioavailability studies and may complement chemical extraction methods.

Biological Tools for assessment of contaminant bioavailability

The complexity of soil, contaminants and receptors (humans, ecosystems, waterways, buildings etc) means that it is impossible to have a "one size fits all" biological test for assessing pollutant bioavailability in soil. A range of factors must be considered when using biological test methods including:-

1. Does the contaminant cause a short-term acute, lethal response (poisoning) or is it a long-term chronic, mutagenic, endocrine disruptive or reproductive failure effect? What actually is an important effect (e.g. poisoning or population crash)?
2. Is a biological test being used to sense a compound or to assess toxic response?
3. Is the test to be used a multicellular organism, a single celled organism or a molecular test.
4. Is the study to be at the site (in situ) or can samples be taken to the lab (ex situ)?
5. What is the assay representing and trying to protect? Human risk? Ecosystem health?
6. Is the test to be in direct contact with the soil (solid phase) or will an extract technique be used? If an extract is used, how relevant is it?

Of the tests discussed here, some are currently used with soil, and some are used for purposes such as sediment testing but could be adapted for use with soil extracts. Some are used for testing toxicity of pure compound or water samples, but again may be adapted for use with soil or soil extracts. The relevance of the biological assay will also depend on the relevance of any extract technique used.

Toxic effects can be categorised as systemic (organism biochemistry and histopathology changes), reproductive, genotoxic or population effects (e.g. abundance and diversity of arthropods, Borras & Nadal, 2004). Methods available are discussed here using a hierarchy of (organism) size to subdivide the topic.

Multicellular organism tests

Tests using whole animals/plants have been used for several decades and as such tend to have large data sets which lend validity to their usage. Initially designed to test factors such as toxicity of pesticides to plants or sediment toxicity, several methods have been enshrined as International Standards Organisation (ISO) Guidance notes (ISO, 1993; ISO, 1995; ISO, 1998; ISO, 1999a; ISO, 1999b; ISO, 2004) and as the Organisation for Economic Co-operation and Development (OECD) Chemicals Testing Guidelines (effects on biotic systems)(OECD, 1984a; OECD, 1984b; OECD, 2000a; OECD, 2000b; OECD, 2003). They include earthworm acute toxicity, earthworm reproduction, terrestrial plant growth (both monocotyledons and dicotyledons), inhibition of root growth, emergence and growth of higher plants and effects on invertebrate reproduction and survival. The advantage of these tests is that they are directly relevant to the specific species examined and represent in situ conditions i.e. they do not require a soil extract to be made and therefore represent actual pollutant bioavailability to a selected organism in soil over time. However the relevance of a particular test, e.g. on a plant species, is difficult to scale up or down to different parts of the ecosystem. Two reviews that go into greater detail are Hund-Rinke & Kordel, (2003), which looks at bioavailability of metals, and the UK Environment Agency Review of ecotoxicological and biological test methods for the assessment of contaminated land, (Environment Agency, 2003). The UK EA report judges tests on the 5 "R" criteria; reproducible, representative, responsive, robust and relevant. A good example of bioavailability assessment of both organics and metals using multicellular organism toxicity tests is given by Cook *et al.*, (2002). They found that soils with levels of contamination above intervention values, according to chemically based soil criteria, did not generate a toxic response to earthworms (mortality test) or seed germination and root elongation (algal growth inhibition and bacterial luminescence were also examined).

Mammalian tests (rodents, dogs, pigs etc), usually used as a surrogate for human risk assessment, are also reviewed by Hund-Rinke & Kordel, (2003). They summarise that the tests are able to judge any toxic response that soil contaminants may induce, but due to complexity of diet, bioavailable concentrations of contaminants remain unknown. Various other mammalian *in vivo* studies of bioavailability of soil

contaminants are summarised in National Research Council, (2002), Ehlers & Luthy, (2003) and Kelley *et al.*, (2002).

The overall advantages of using multicellular organisms to test for bioavailability are that the tests are directly relevant to the organism used, can show systemic changes, for example reactions to oestrogen like substances, and can be reasonably cheap to carry out (e.g. plant assays). Limitations include no direct representation of other organisms, difficulties translating results (normalising) between different field sites and the time it takes to perform the tests – days/weeks rather than hours. Mammalian tests are further complicated by strict regulations governing animal welfare and expense. Validation of *in vitro* test methods against animal tests involves comparing levels of a contaminant found in blood or urine (e.g. Ruby *et al.,* 1996) to that extracted in the *in vitro* method. Only one study exists that directly tested contaminated soils on humans (Maddaloni *et al.*, 1998) although the ethics of this research are questionable.

A simpler and more ethical technique may be the use of human cell lines for toxicity assays as they may more accurately represent human toxic responses than other organisms. A test that has been used involving eukaryotic organisms (or *in vitro* cell lines) is the single cell gel electrophoresis assay (SCGE), more commonly known as the "Comet" assay (due to the subsequent appearance of cells). It is a sensitive and subtle assay that can be used to quantify and assess DNA damage in single cell preparations of any eukaryotic cells (i.e. cells with DNA in nuclei).

First described by Ostling & Johanson, (1984) and then adapted by Singh *et al.*, (1988) (alkaline comet), the Comet assay is heavily used in applications ranging from genotoxicity testing (of known and new compounds), to human and ecological biomonitoring, to research into DNA damage and repair mechanisms. The comet assay has been utilised in environmental genotoxicology studies (generally reviewed by Cotelle & Ferard, 1999) including investigations into the effects of soil contaminants in organisms exposed to soil.

Whole cell test organisms

The past two decades have seen an explosion of microbial/whole cell tests for use in estimating contaminant bioavailability. Genetic engineering and modification techniques have allowed indicator genes to be coupled to genes of specific interest to give a qualitative and quantitative response. The first use of a reporter gene to show a phenotypic response was the Ames test (Ames, 1973). This showed whether a compound caused a mutagenic response by causing a reversion from histidine dependence i.e. if a mutagenic compound was present the *Salmonella typhimurium* would grow without additional histidine. Although not directly tested with contaminated soil, it is still suitable to use with soil extracts. The extract used (water/solvent/buffer etc) is as important as the biological test chosen when assessing the toxicity of a contaminated soil as the nature of the extract will be the factor that determines bioavailability. The Ames test and its successors are reviewed by Wegrzyn & Czyz, (2003). These mutagenic biosensors tend to be sensitive, reasonably quick to perform (days or hours, not weeks) but are limited by the need for specialist (expensive) equipment and their relevance to other organisms.

The best established microbial test in environmental testing is the Microtox assay (www.azurenv.com). A bioluminescent marine bacterium, *Vibrio fischeri,* produces light as a by-product of normal cell functions. Any toxicity inhibits cell functions and proportionally, light emission, allowing toxicity to be quantitatively measured. Microtox itself is still widely used and has been shown to be highly sensitive, more so than various aquatic multicellular organism tests (Munkittrick *et al.*, 1991). Advantages include sensitivity in its general toxic response, simplicity and rapidity, robustness and reproducibility and the sheer amount of data produced using it. Major limitations are that it relies on exposure to an extract, which may present difficulties in interpreting results and is a marine organism and therefore not strictly relevant in its response to soil contamination.

The principle behind Microtox, of light emission, has given rise to huge number of genetically engineered bacteria, yeast and mammalians cells which use light as an indication of bioavailability. "Lights off" systems, like Microtox, reduce light emission in response to general toxicity. "Lights on" systems, like Mutatox, have a

luminescent gene coupled to a specific reporter gene (e.g. a stress response gene like rec A) or one that can detect specific classes of pollutants (e.g. metal sensitive biosensors, Rensing & Maier, 2003 or nutrients Joyner & Lindow, 2000) and emits light when suitably provoked. Microbial biosensors are eloquently reviewed in greater detail by Hansen & Sorensen, (2001), Leveau & Lindow, (2002) and Belkin, (2003). Several UK biotechnology companies have emerged (for example www.remedios.uk.com and www.cysense.com) that use luminescent bacteria specifically to assess contaminated land.

The reviews by Leveau & Lindow, (2002) and Belkin, (2003) also discuss similar reporter gene based systems that use ß-galactosidase/lac Z and Green fluorescent protein (GFP). The former was originally used in the SOS chromotest (Quillardet *et al.*, 1982) an Ames-like test. The latter is now equally as popular as luminescent based tests, using fluorescence rather than luminescence. For example, Knight *et al.*, (2004) presented a yeast/GFP genotoxicity assay used to test a range of environmentally relevant substances (pesticides, metals, solvents). Biosensors that utilise both luminescence and fluorescence, in order to test for acute and genotoxic threats simultaneously, are also available. The most high profile usage of these tools is as health monitors in the International Space Station (Rabbow *et al.*, 2003).
Advantages of these light/colour based toxicity indicator tests are ease of assay, speed of assay, versatility and sensitivity, up to a point – currently chemical analysis is more sensitive but that may change. Disadvantages are a lack relevance to other (multicellular) organisms, exposure to soil extracts rather than soil itself (soil extracts may not represent the actual in situ bioavailability of the pollutant) and, probably the factor most difficult to overcome, that these organisms have been genetically modified makes use beyond the lab (i.e. on site) awkward, if not impossible due to safety regulations (HMSO, 1990; HMSO, 2002).

The Environment Agency (Environment Agency, 2003) has recommended the use of reverse transcriptase PCR to measure gene expression as a tool for ecological risk assessment. It is a method that may be used to look at thousands of genes at once (and consequently compare species response) and their responses to vast arrays of contaminants. Sturzenbaum et al (Sturzenbaum *et al.*, 1998a; Sturzenbaum *et al.*, 1998b; Sturzenbaum *et al.*, 2001) examined changes in gene expression (in

earthworms exposed to contaminated soil) in metallothionein, carboxypeptidase and other metal sensitive genes and found transcription levels up to 100 fold greater in exposed organisms showing that the technique has a high degree of sensitivity. A great advantage of this technique is that organisms that have been directly in contact with contaminated soil can be analysed and therefore bioavailability is the parameter being assessed. A disadvantage is determining what "normal" levels of gene expression are in order to determine whether contamination has had an effect. It is also important to differentiate between 'normal stress responses' e.g. to drought, and those actually related to pollution. Microbial and whole cell biosensors may be best employed as initial screening tools for environmental and soil contamination.

Sub-cellular or molecular tests

Rather than engineering reporter genes into cells it is possible to look directly at the molecular system of interest. Biosensors in common use for environmental and food contaminants include nucleic acid based (e.g. DNA hybridisation array, Fredrickson *et al.*, 2001), enzyme based and antibody and receptor based. All these categories are reviewed in Baeumner, (2003).

Antibody interactions are also highly sensitive and very specific. Immunoaffinity has been adapted from clinical research to quantify environmental pollutants like DDT and its breakdown products (Anfossi *et al.*, 2004), metals (Chavez-Crooker *et al.*, 2003), dioxins (Okuyama *et al.*, 2004) and many others. Antibodies can be customised and raised against any contaminant and are supplied by various biotechnology companies e.g. www.remedios.uk.com. The relevance of any antibody assay for bioavailability purposes will be dependant on what soil extraction method is used.

Assays that measure levels of DNA adducts (regarded as the first step in mutagenesis) have been used to determine the effects of exposure of earthworms to PAH's. Short term exposure gave levels of DNA adducts that were dose dependant (Saint-Denis *et al.*, 2000). The potential use of this method to analyse soil DNA to assess in situ bioavailability is described in Singleton & Lyons, (in press). These *in vitro* bioassays may be limited by their general applicability to organisms or ecosystems (and high cost of development) but in general due to their speed of assay (hours instead of days/weeks) they are very useful as initial bioavailability and toxicity screening tools.

1.8 Summary and aims of the project

In summary, whilst there are estimates of the extent of land contamination in the UK and internationally, there are few definitive figures and it is likely that many urban and industrialised areas are heavily contaminated with a wide range of pollutants. The potential risk this contamination poses to public health is also unquantified and therefore there is a need to determine urban levels of contamination. Current regulatory regimes are limited to chemical analysis and soil guideline/trigger values, above which other currently unspecified methods of assessment can be employed to further investigate whether the contaminated soils do pose a human health risk.

The concept and practise of using bioavailability investigations, more particularly simulated oral bioavailability methods, to assess risk associated with contaminated soils is a growing area of research internationally. These chemical assessment methods are being developed as there are currently no experimental techniques available for directly testing the toxicity of contaminated soils on humans and animal surrogate tests are expensive, time consuming and ethically problematic.

There is a need for a biological assay that is cheap, quick, robust and more relevant to human toxicity assessment. It is also recognised that bacterial assessment of contaminated soils can be useful, either as a preliminary screening tool for human toxicity or as a representative of specific soil ecosystems.

Therefore the aims of this project were as follows:-

1. To determine if a waste incinerator in urban Tyneside (Byker incinerator) had contributed to soil pollution in the surrounding area.
2. To determine the extent of soil contamination in an urban UK environment specifically examining levels of dioxins, arsenic and heavy metals, using Newcastle upon Tyne (and greater Tyneside) as an example.
3. To further investigate the risks any contaminated soils may pose to human health using a range of chemical extraction methods that estimate bioaccessibility.
4. To develop a novel and relevant human cell *in vitro* toxicity assay to further investigate the biological risk contaminated soils may pose to human health.
5. To develop and optimise a rapid bacterial bioassay using a native soil organism in order to further investigate and assess the biological risk contaminated soils pose to ecosystems.

Chapter 2 – Investigation into soil contamination in urban Tyneside around the Byker incinerator

2.1 INTRODUCTION

2.1.1 Urban soil contamination - research into soil levels of arsenic, heavy metals and dioxins around incinerators

The public perception of incinerators, especially those in urban and suburban areas, is that they pollute surrounding areas and may pose a health risk to the general public. Here the effect that the Byker incinerator in Newcastle has had on the pollutant burden in surrounding soils is examined. As discussed in the main introduction, the make-up of waste that is burnt in an incinerator affects the content of any discharges (Williams, 1994); the most common emissions include metals and dioxins. Toxic effects that these contaminants cause include both acute and chronic responses including carcinogenesis, reproductive problems, developmental impacts and damage to neurological, haematopoietic, renal and hepatic function.

With the improvement in technologies, metals in stack emissions (except Hg) have dropped (Allsopp *et al.*, 2001) over the past 10 years, although emissions may previously have added to the soil pollution burden over time.

Several studies have investigated the effects of incinerators on soil contamination, most commonly examining dioxin levels. Nadal *et al.*, (2002) sampled soil and herbage before (1998) and after (2000) technical improvements were made to a municipal solid waste incinerator (MSWI) in Montcada, Spain and determined that the incinerator was not the main source of environmental PCDD/F in the area. Nadal *et al.*, (2005) also sampled soil and herbage over time (1998, 2001 and 2003) around a hazardous waste incinerator (HWI) in Constanti, Spain, for a range of metals (As, Be, Cd, Cr, Hg, Mn, Ni, Pb, Sn, Tl and V). Over the sampling time, some metal concentrations in soils rose (As, Be, Cr, Ni and V) whilst some decreased significantly (Cd, Hg and Sn). The influence of the

incinerator on metal concentration fluctuations was found to be minimal in relation to other sources of pollution in the area.

Park *et al.*, (2004) found that levels of dioxins in soil and human blood (in South Korea) were not influenced by proximity to an industrial waste incinerator but when congener (homologue) patterns were analysed changes due to distance were clearly seen. Capuano *et al.*, (2005) examined dioxins, PCB's, PAH's and metals from a municipal solid waste incinerator (MSWI) and found dioxins, PCB's and PAH's to be low (below Italian guideline values) and metal concentrations to be related to either the local geology or sources other than the incinerator. Caserini *et al.*, (2004) examined levels of dioxins in air and soil samples around 3 MSWI's with different dioxin removal systems; the first showed no increase in dioxins (range 0.7-1.5 ng/kg I-TEQ soil) over the 2 years of operation, the second was not equipped with the best available dioxin removal showed levels between 1.1 and 1.4 ng/kg I-TEQ in soil and the third showed levels of dioxin from 0.08 to 1.2 ng/kg I-TEQ.

Numerous studies into the effect of incinerators on soils in the UK, and studies looking at general levels of arsenic, heavy metals and dioxins in UK soils have been carried out. The available data from several studies is summarised in Table 2.1 and gives an indication of what levels would be considered "normal" in UK soils.

Table 2.1 Levels of As, heavy metals and dioxins found in soils in the UK in previous studies. Data supplied as a range or mean ND=no data.

Study/location	As (mg/kg)	Cd (mg/kg)	Cr (mg/kg)	Cu (mg/kg)	Hg (mg/kg)	Ni (mg/kg)	Pb (mg/kg)	Zn (mg/kg)	Dioxins (in ng/kg I-TEQ)
Hampshire (4 incinerators) (Abbott *et al.*, 1997a; Abbott *et al.*, 1997b).	2-18	0.1-5.7	10-200	ND	0.1-2.8	4-87	16-8300	ND	2.2- 160
Hampshire (background) (Abbott *et al.*, 1997a; Abbott *et al.*, 1997b).	5.5-8.8	0.3-1.0	Mean=20	ND	0.1-0.2	5.3-32	24-29	ND	1.7-3.8
Dudley (incinerator) (Allan *et al.*, 1999).	3-29	<0.5-3.0	15-64	ND	<0.2-1.2	8-44	33-430	<66-680	ND
Newcastle (sediments) (BGS, 1993).	14-15	1-1.2	100-150	20-30	ND	50-60	70-300	200-600	ND
Newcastle (gardens and allotments)(Thornton, 1985).	ND	1.4-1.8	ND	ND	ND	ND	840-1608	506-660	ND
England and Wales (McGrath, 1987; McGrath & Loveland, 1992).	ND	0.7-40.9	Mean=34	Mean=23	ND	Mean=26	ND	ND	ND
UK (HMIP, 1995).	ND	ND	ND	ND	ND	ND	ND	ND	Rural 0.8-17.5 Urban 4.9-87.3

2.1.2 Research into soil contamination in urban Newcastle upon Tyne and Tyneside.

The long and varied industrial history of the Tyneside area has led to previous investigations into the state of the local soils. In 1988, Aspinall *et al.*, (1988) sampled the whole Tyneside area on a km grid basis. Samples (412) were taken from public open spaces and analysed for both total and plant available levels of cadmium, lead and zinc. Sixty nine % of samples were found to have total lead above 80 mg/kg with 35.4% with concentrations between 150 and 550 mg/kg and 6.5% over 550 mg/kg. Total zinc was found to be over 300 mg/kg in 14.4% of samples and total cadmium over 1 mg/kg in 47.4% of samples.

Mellor, (2001) sampled topsoil and stream sediments in the Wallsend Burn (a stream several miles east of Byker) and analysed for total and plant available lead and zinc. 121 topsoil samples were taken and in addition to metal analysis, pH and organic content of each sample was tested for. The mean total Pb was found to be 129 mg/kg, and Zn to be 282 mg/kg. The organic content mean was 18% and the pH mean was 6.1.

Gamble, (2001) investigated the influence of the Byker incinerator, on a smaller scale than this study. 54 samples were taken within a radius of 1800 m from the plant with 6 control samples taken from outside the study area. Total cadmium, copper, iron, manganese, lead and zinc concentrations were investigated, as were pH and organic matter %. Data was presented graphically (not numerically) and the conclusions drawn were that there were sites in Byker where metal concentrations exceeded toxicity thresholds but the source(s) was not the incinerator.

2.1.3 The Byker project

In autumn 1999, the incinerator plant in Byker, Newcastle upon Tyne, became the focus of concerns from local residents about potentially toxic fly ash from the site that had been mistakenly used (between 1994 and 1999) to stabilise pathways in a range of places (mainly allotment paths) around the city. An initial

report was commissioned by Newcastle City Council (in conjunction with Newcastle and North Tyneside Health Authority) to investigate any potential health hazards. The report (Pless-Mulloli *et al.*, 2000) recommended further investigations into, not only the sites affected by the ash, but sites within the potential sphere of influence of the incinerator. This included soil around the affected allotment paths, vegetables grown in affected allotments, eggs from the allotments, soil from the Walker road allotment site (directly to the east of the incinerator) and soils within the span of the incinerator plume (subject of this chapter). In relation to this end, an application was made to DEFRA to address residents concerns that the incinerator had contributed to soil pollution. This stemmed from the residents observations of incinerator emissions being deposited in the local area. DEFRA money was made available to assess both soil heavy metal and dioxin levels around the incinerator. For each project, a steering group that included local residents, City Council officers, Health Authority members and University researchers was formed – referred to in this report as the "Byker Ash steering group".

2.1.4 Aims

The main aims of this investigation for the Byker ash steering group were

a) to assess whether the Byker incinerator had contributed to the pollutant burden of soils from fugitive and stack emissions in the vicinity of the plant (i.e. in the surrounding Tyneside area), specifically examining dioxin, arsenic and heavy metal levels
b) to advise Newcastle City Council regarding further action

This study was useful in that it enabled additional assessment of the extent of soil contamination in urban Tyneside (with less emphasis on attribution of responsibility for any contamination). Additional aims of this chapter were:-

1. To assess the extent of dioxin contamination in urban Tyneside soils.
2. To assess the extent of heavy metal and arsenic contamination in urban Tyneside soils.

2.2 METHODS AND MATERIALS

The location of the Byker plant, within the UK, is shown in Fig. 2.1

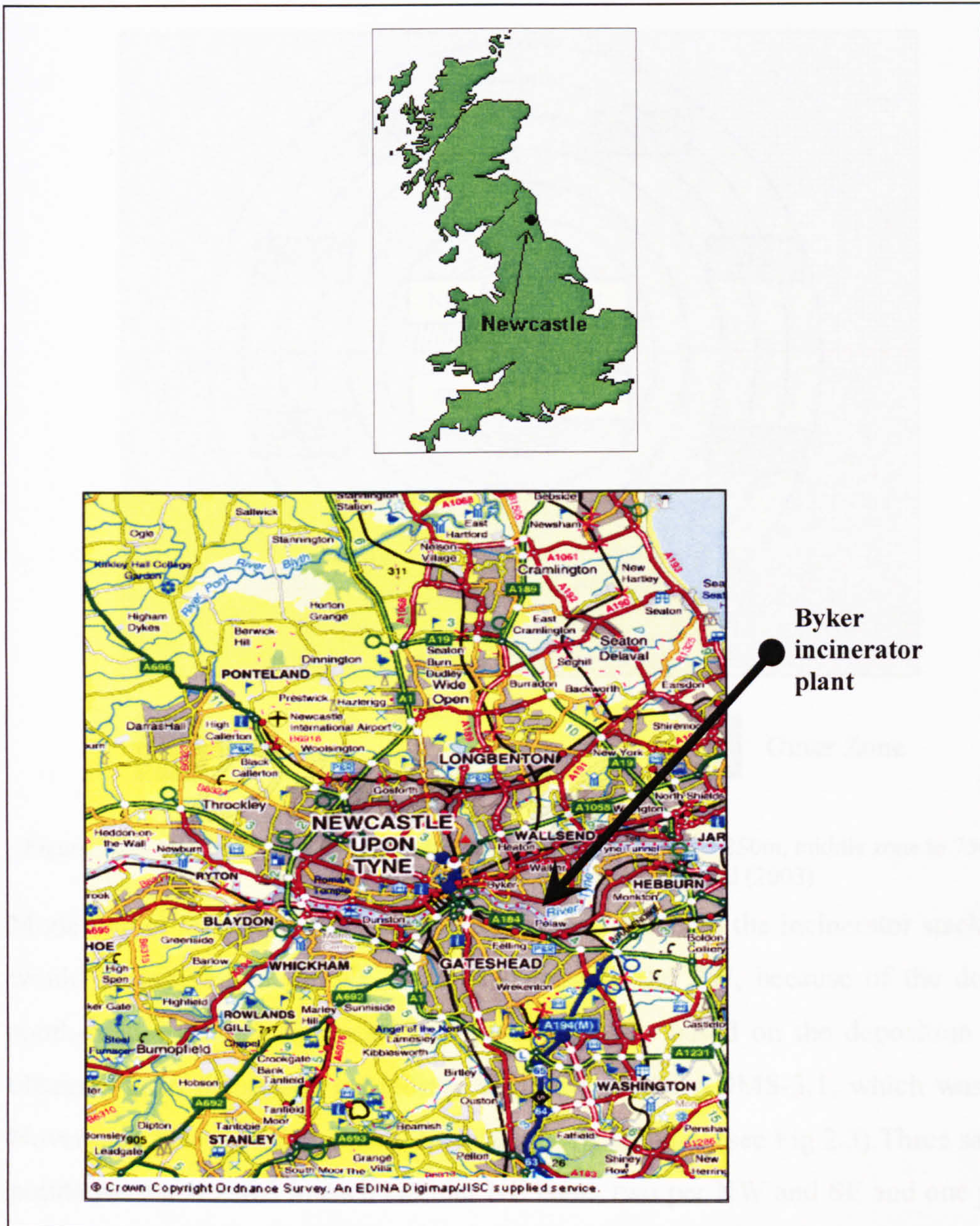


Figure 2.1 Study location map

2.2.1 - Soil sampling regime

The area around the Byker plant (using the incinerator chimney as the central point) was divided into four main sections, north east (NE), north west (NW), south east (SE) and south west (SW) up to a radius of 2250m with further subdivision into 50m bands.

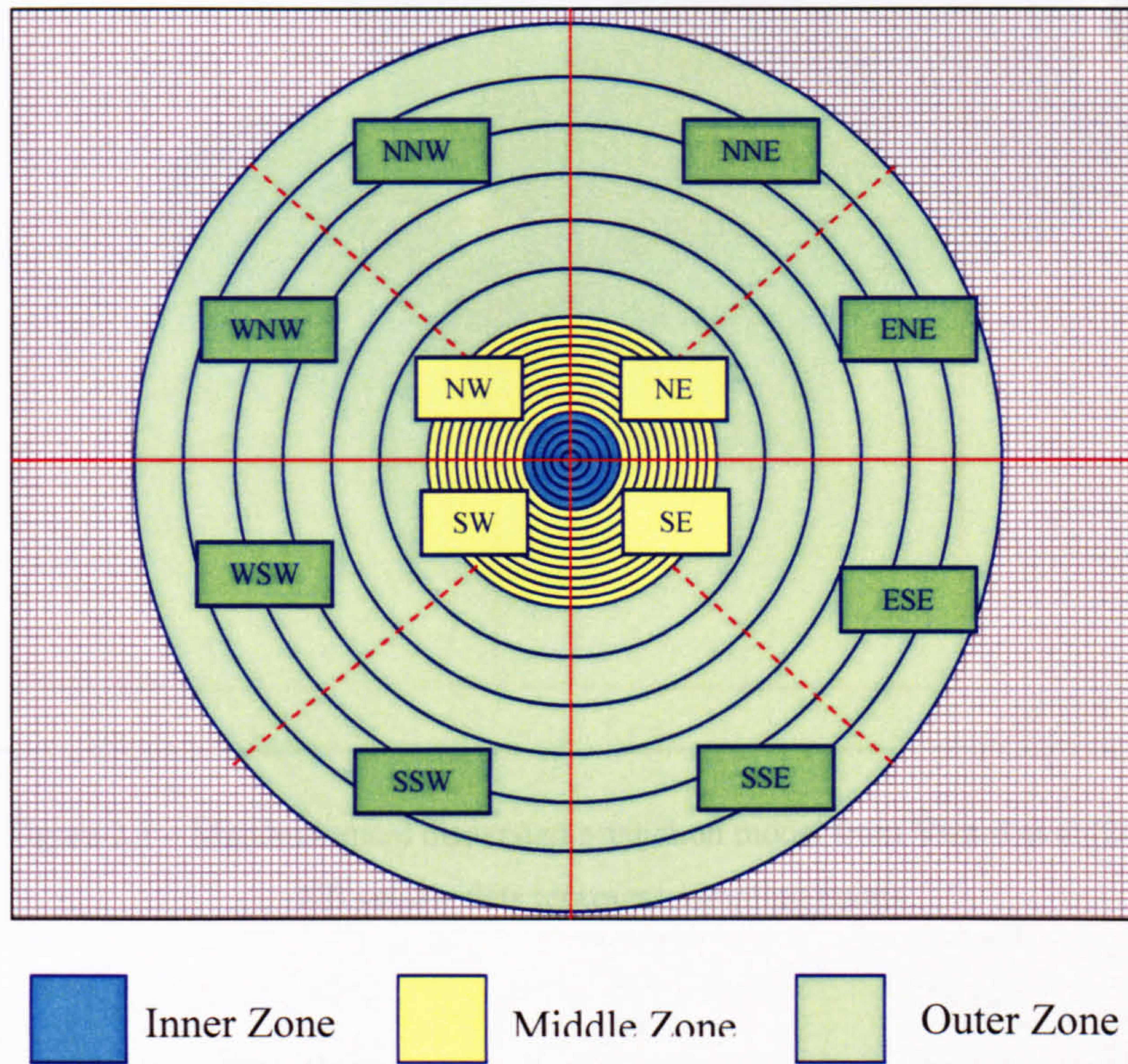


Figure 2.2 – Map of the "zones" around the Byker plant (inner zone 250m, middle zone to 750m and outer zone to 2250m)– taken from Vizard et al (2003)

Modelling of meteorological data predicted that effects of the incinerator stack plume would be greatest in the NE sector and least in the SW, because of the dominant south-westerly wind direction. This prediction was based on the deposition pattern obtained from dispersion modelling, using the model ADMS-3.1, which was run in November 2001 by Mike Elund, Newcastle City Council (see Fig 2.3).Three sampling points were generated in each circular NE band, two per NW and SE and one per SW band (see Fig 2.3). In total 163 sites were sampled. Between 4 and 8 sub-samples were taken from a square of up to 50 m drawn around each sampling point. Three soil samples were taken (using steel cylinder cores of 5 cm by 5 cm) at each sub-sample point. One core from each sample point was added to a "pooled" sample per site. Samples were stored in dark glass jars at 4°C until analysis.

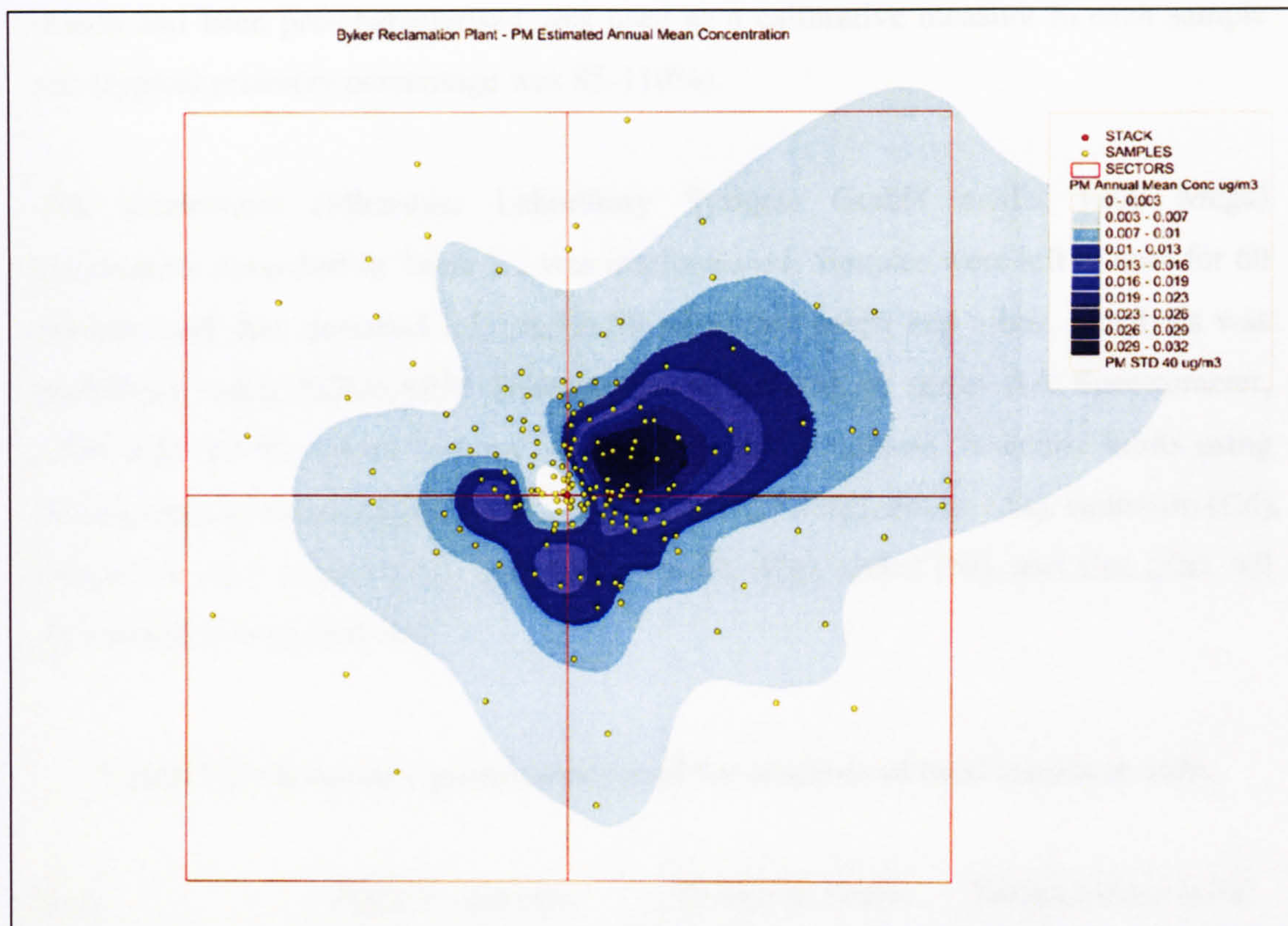


Figure 2.3 – Meteorological dispersion prediction model from Vizard et al (2003).
NB yellow dots represent sampling points

2.2.2 Sample preparation

All samples were air dried on aluminium foil dishes for 1 week, plant material removed manually and the soil ground in a marble pestle and mortar. Soil was then sieved to <2 mm (sieve ISO 3310-1 approved 2mm) and stored in glass jars at ambient temperature. This fraction was used in all further experiments.

2.2.3 Total metal analysis

0.5 g of sample was weighed into a quartz jar, 8 ml of nitric acid (HNO_3, Suprapur Nitric acid 65% 1 L, Merck) and 2 ml hydrogen peroxide (H_2O_2, 30% perhydrol proanalysis 1L, Merck) were added and a loose quartz lid placed on top. The jar was placed into a microwavable plastic capsule with 6 ml of deionised water and 2 ml H_2O_2 in the bottom. Reference material 207 (SETOC sample 755, 150 g, sediment 43 Norway, Wageningen evaluating programme for analytical laboratories, Netherlands)

which had been pre-characterised was used as a calibrative measure in each sample run (typical recovery percentage was 85-110%).

The microwave (Micromar Laboratory Systems GmbH model 1200 Mega) programme described in Table 2.2 was implemented. Samples were left to cool for 60 minutes and then decanted into sterile 50-ml plastic screw cap tubes. Analysis was performed using SOLAARM Datenstation, UNICAM M series AA Spectrometer, UNICAM VP 90 vapour systems and GI 95 graphite furnace (detection limits using this equipment were in ppb). Elements analyzed for were: arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn). All 163 samples were analysed.

Table 2.2 Microwave programme used for analysis of total metals in soils.

Step	Time in minutes	Power in Watts	Temperature in °C
1	5	250	80
2	1	0	80
3	5	400	80
4	3	0	80
5	5	600	80
6	3	0	80
7	3	700	80
8	5	250	80

Results generated and reported in 2.3.1 were single values per sample (i.e. no replicates) due to analysis taking place in a commercial laboratory where this is standard practice.

2.2.4 Dioxin and furan (PCDD/PCDF) analysis

NB – All methods used were from the standard operating procedures of ERGO Forschungsgesellschaft mbH, Hamburg, Germany – a laboratory accredited according to ISO 17025 and DIN EN ISO/IEC 17025 (ISO, 1999c).

Extraction from soil

One g of dried sample was weighed into an extraction thimble (pre-treated with methanol and toluene) and spiked with 200 μl of preparation standard (added as a ^{13}C recovery efficiency standard, see Table 2.3). 4 g of fine sodium sulphate ($NaSO_4$, Merck, >8hrs at 500°C pre-treatment) was added and mixed in with the soil to absorb any remaining water. The thimble was plugged with glass wool and inserted into a 30 ml glass soxhlet tube (all glassware was pre-rinsed with toluene and hexane). This in turn, was inserted into a 100 ml round bottom flask containing 80 ml toluene (Baker) and 3-5 Teflon boiling stones. The flasks were then placed on a boiling bank for 20 hours. Acetone was used to rinse the boiling bank tubes to ensure all sample extract washed into the flask. The flasks were then rotated on a rotary evaporator (Buchi REII) at 330-250 mbar and 40°C water bath temperature, until dry.

Purification of dioxins from soil

a) *"Clean-up" of soil extract*

30 ml of hexane (Merck, Suprasolv, 2.5 L), 4 g (accuracy of 0.1 g) of H_2SO_4/SiO_2 powder (Baker and Fluka respectively, 240 g SiO2 : 160 g H_2SO_4, SiO_2 treated for 20 hrs in a soxhlet with methanol and 20 hrs with dichloromethane, dried with N_2 then 12 hrs at 140°C) and 2 g (accuracy 0.1 g) K/SiO_2 powder (Merck, 224 g KOH + 950 ml methanol, 400 g SiO_2, 2 hrs at 55°C on rotary evaporator, stand overnight, wash with 4 L methanol and 6 L dichloromethane) was added to the flask and left to stand overnight.

b) *Ergo column*

The flask was then rinsed with hexane which was poured into a glass chromatographic column (250 mm x 22 mm, ergo column) plugged with glass cotton (GC, Shimadzu), the liquid was collected in a 100ml pear shape flask. The column was continuously washed with hexane until 80-90 ml had collected in the new flask. This was then rotated on the rotary evaporator at 330-250 mbar and 40°C water bath temperature, until dry.

Table 2.3 Preparation standard SM IX

Congener	^{13}C – concentration in ng/ml
2,3,7,8 TCDD	5
1,2,3,7,8 PeCDD	5
1,2,3,4,7,8 HxCDD	5
1,2,3,6,7,8 HxCDD	5
1,2,3,7,8,9 HxCDD	5
1,2,3,4,6,7,8 HpCDD	5
OCDD	50
2,3,7,8 TCDF	5
1,2,3,7,8 PeCDF	5
2,3,4,7,8 PeCDF	5
1,2,3,4,7,8 HxCDF	5
1,2,3,6,7,8 HxCDF	5
1,2,3,7,8,9 HxCDF	5
2,3,4,6,7,8 HxCDF	5
1,2,3,4,6,7,8 HpCDF	5
1,2,3,4,7,8,9 HpCDF	5
OCDF	5

(recovery efficiency standard)

c) Tandem-Alox column

An Alox column, plugged with GC, was filled with 3.65 g Alox (ICN biomedical, > 20 hr 180°C, maintained at 180°C until use) with a layer of 0.5 g fine $NaSO_4$ on top. This was flushed with hexane, forced through with N_2 (Westfalen gas 2 bar pressure), until no air was left in the powder. A 230 mm pasteur pipette, plugged with GC was filled with 0.75 g of H_2SO_4/SiO_2 with a layer of 0.35 g of Cs/SiO_2 (Aldrich chemicals CsOH 250 g +835 ml methanol + 420 g SiO_2, stand overnight, wash 4 L methanol, 6 L dichloromethane, dry with N_2). This was flushed with hexane in an identical fashion to the tandem column and then placed above the tandem column. The pear shaped flask was rinsed with 4 x 2 ml hexane which was

then added to the tandem column. The tandem column was then removed. 7 ml hexane and 6 ml 98:2 Hexane: Dichloromethane (LGC Promochem, Picograde, 4 L) were then dripped through and the solvent waste then disposed. Twenty five ml of 1:1 cyclohexane: dichloromethane were then washed through the column and collected in a 25 ml pear shaped flask. The flasks were again placed on the rotary evaporator until dry.

d) Carbopac column

A Carbopac column, plugged with GC was filled with 0.5 g Carbopac powder (Supel Co, 80-100 mesh, 18 carbopac: 82 coalite, extracted in toluene soxhlet 2 x 20 hr) which in turn was covered with GC. The column was then filled and flushed through with toluene (LGC Promochem, Picograde, 4L) 3 times. 3 x 2 ml 1:1 cyclohexane: dichloromethane was used to rinse the flask and then added to the column. 1 ml 1:1 cyclohexane: dichloromethane was added. 80 ml toluene was then dripped through and collected in a 100 ml pear shaped flask. This was again rotated until dry at 70-50 mbar and 40°C.

e) Tandem column

This was made up as previously described in c). 4x 2 ml hexane was used to rinse the flask and was dripped through the column into a 10 ml pear shaped flask. This was again rotated until dry at 70-50 mbar and 40°C.

f) Inserts

Using a needle pipette, 100 µl hexane was used to rinse the sides of the flask and then injected into a microinsert (M + N art no 702818 ~20µl capacity). This was concentrated by drying with N_2. The process was repeated twice and 10 µl syringe standard (10 ng 1,2,3,4 – ^{13}C, TCDD/ml) was then added. The inserts were sealed in sample vials (M + N art 70201) with aluminium caps (M + N art 70231) and then stored at 4°C until analysed using HR-GC/MS.

HR-GC/MS

NB - The analysis was carried out in full by trained ERGO Forschungsgesellschaft mbH personnel using an internal protocol.

High resolution gas chromatography and high resolution mass spectrometry (HR-GC/MS) in combination with VG-Autospec-Finnigan MAT 95 XL on DB 5 (non-polar and SP2331 (polar) capillary columns was used to analyse for PCDD/PCDF. Injection volume was 2 µl at 270°C and 185 kPa (HRGC). Column specific temperature and time programs are shown in Table 2.4. HRMS temperature in the ion source was 280°C.

Table 2.4 Temperature and time specific parameters for DB5 and SP2331 chromatography columns.

	DB5	SP2331
Starting temp in °C	90	90
Isothermal	3 mins at 90°C	3 mins at 90°C
Heating rate 1	25°C/min to 210°C	25°C/min to 210°C
Heating rate 2	3°C/min to 275°C	3°C/min to 250°C
Isothermal	20 mins at 275°C	65 mins at 250°C

Due to cost restrictions only 82 of the 163 samples were analysed for dioxins. . The samples chosen for analysis were systematically selected from alternating sample points i.e. from the 50, 100, 200, 300, 400, 500, 600, 700, 1000, 1500 and 2000 m bands of all 4 sectors. Results generated and reported in 2.3.2 were single values per sample (i.e. no replicates) due to analysis taking place in a commercial laboratory where this is standard practice.

Note on responsibility for work carried out

The larger "Byker project" investigating the role of the incinerator involved many people over several years. The work that I personally carried out (which is described in this thesis) included attending planning meetings, the vast majority of the soil sampling (2.2.1), all of the sample preparation (2.2.2), all of the metal analysis (2.2.3) and all of the dioxin/furan analysis, excepting the HR-GC/MS (2.2.4).

2.3 RESULTS

2.3.1 Arsenic and heavy metal levels in soil samples

The full data set is available in Appendix 2, summary statistics in Table 2.5 and a graphic showing the percentage of samples exceeding the guideline value in Fig. 2.1.4.

Arsenic

Levels ranged from 5 - 279 mg/kg (n=163) with 52 samples exceeding the CLEA SGV of 20 mg/kg for residential areas and allotments; no samples were over the 500 mg/kg CLEA guideline value for industrial areas. Levels are shown by sector and distance band in Fig. 2.4.

Cadmium

Levels ranged from 0.01 - 6.95 mg/kg (n=163) with 36 samples above the CLEA guideline value for pH 6 (1 mg/kg) (evenly distributed by sector with the highest levels in the SW sector); of the 36, 7 also exceeded the guideline value for pH 7 (2 mg/kg). No samples exceeded the pH 8 guideline value (8 mg/kg). Levels are shown by sector and distance band in Fig 2.5.

Chromium

Levels ranged from 23 - 230 mg/kg (n=163) with 3 samples greater than the CLEA guideline value of 130 mg/kg for residential areas with plant uptake (in the SE and SW sectors); 2 of the 3 samples also exceeded the 200 mg/kg CLEA guideline value for residential area without plant uptake (both in the SE sector). Levels are shown by sector and distance band in Fig 2.6.

Copper

Levels ranged from 20 - 12,107 mg/kg (n=163). As there is currently no CLEA guideline value for Cu, the ICRCL trigger value of 130 mg/kg was used as a guideline. Although the ICRCL trigger values were based on phytotoxicity risk (not human health risk) these were the only values available to use as a guideline. 39 samples exceeded 130 mg/kg with the highest levels in the SE and SW sectors. Levels are shown by sector and distance band in Fig. 2.7.

Mercury

Levels ranged from 0.03 - 4.99 mg/kg. The most stringent CLEA guideline value for residential areas with plant uptake is set at 8 mg/kg – no sample exceeded this value. Levels are shown by sector and distance band in Fig. 2.8.

Nickel

Levels ranged from 11 - 165 mg/kg with 9 samples exceeding the CLEA guideline value for residential areas with plant uptake of 50 mg/kg (NW, SW and SE sectors); of the 9, 6 samples also exceeded the 75 mg/kg level set for residential areas without plant uptake (NW and SW sectors). Levels are shown by sector and distance band in Fig. 2.9.

Lead

Levels ranged from 40 – 4,134 mg/kg with 27 samples exceeding the CLEA guideline value of 450 mg/kg for residential areas. 13 of the samples also exceeded the 750 mg/kg guideline value for industrial sites. The highest levels were seen in the SE and SW sectors. Levels are shown by sector and distance band in Fig. 2.10.

Zinc

Levels ranged from 75 – 4,625 mg/kg with the highest levels seen in the SW sector. As with Cu, no CLEA guideline exists; using the ICRCL trigger value of 300 mg/kg as a guideline. Although the ICRCL trigger values were based on phytotoxicity risk (not human health risk) these were the only values available to use as a guideline. 70 sites were found to be over guideline. Levels are shown by sector and distance band in Fig. 2.11.

Table 2.5 Summary statistics of levels of heavy metals and arsenic and dioxins in soils in urban Tyneside

mg/kg	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn	Dioxins (ng/kg I-TEQ)
Mean	20.11	0.34	55.19	233	0.50	29.59	350.05	419	116.12
Median	14.67	0	50.95	76.55	0.32	25.66	233	273.63	28.85
Minimum	4.75	0	23.10	19.98	0.03	10.50	40.28	74.60	5.98
Maximum	279	6.95	230	12107	4.99	165	4134	4625	1911
No. sites over soil guideline value (CLEA, ICRCL and various)	51	15	3	39	0	12	27	70	3

Arsenic

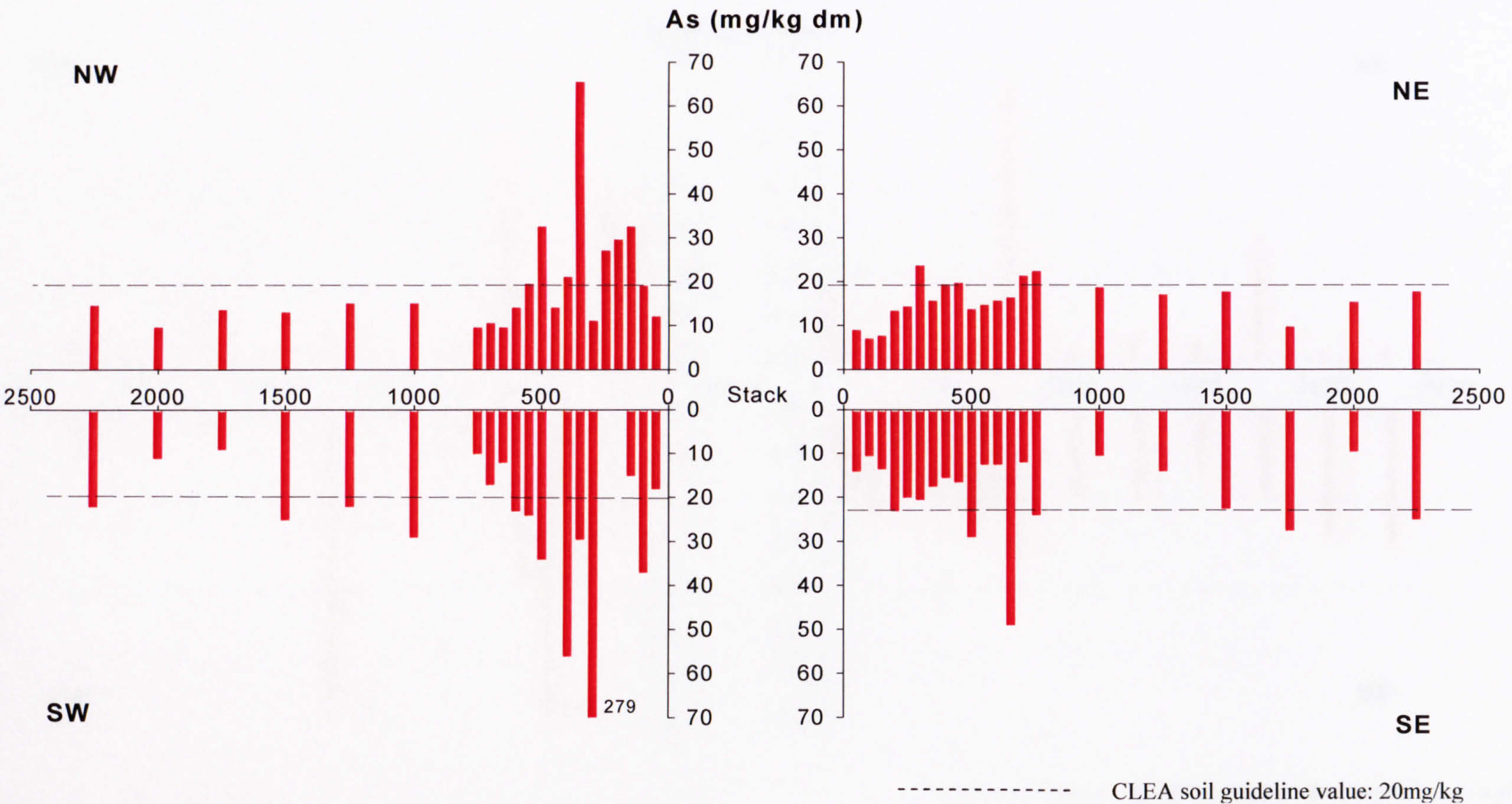


Figure 2.4 Mean soil arsenic levels (mg/kg) by sector and distance band NB mean values in NW, SE, NE sectors; single readings in SW sector. Adapted from Vizard et al (2003).

Cadmium

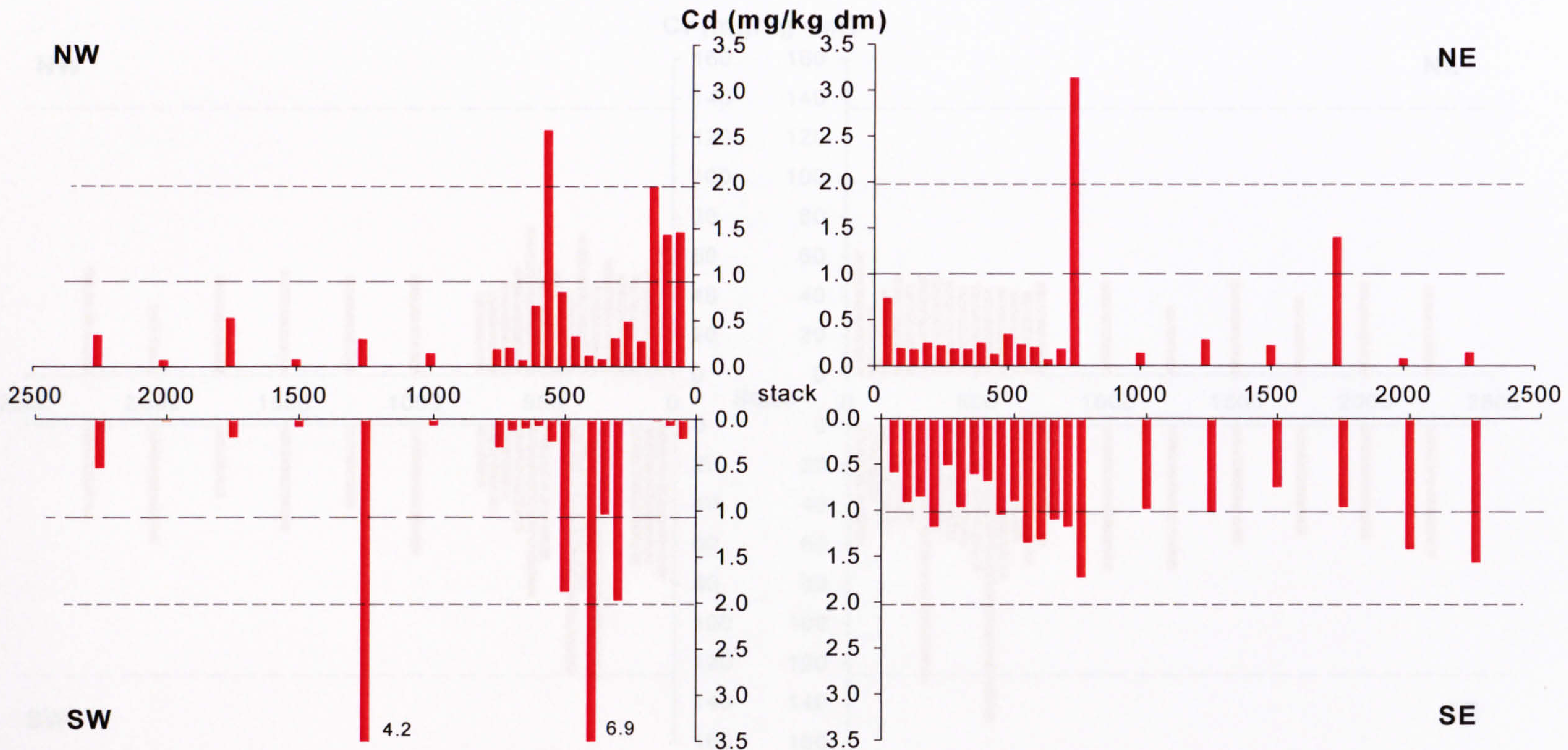


Figure 2.5 Mean soil cadmium levels (mg/kg) by sector and distance band. Adapted from Vizard et al (2003).

Chromium

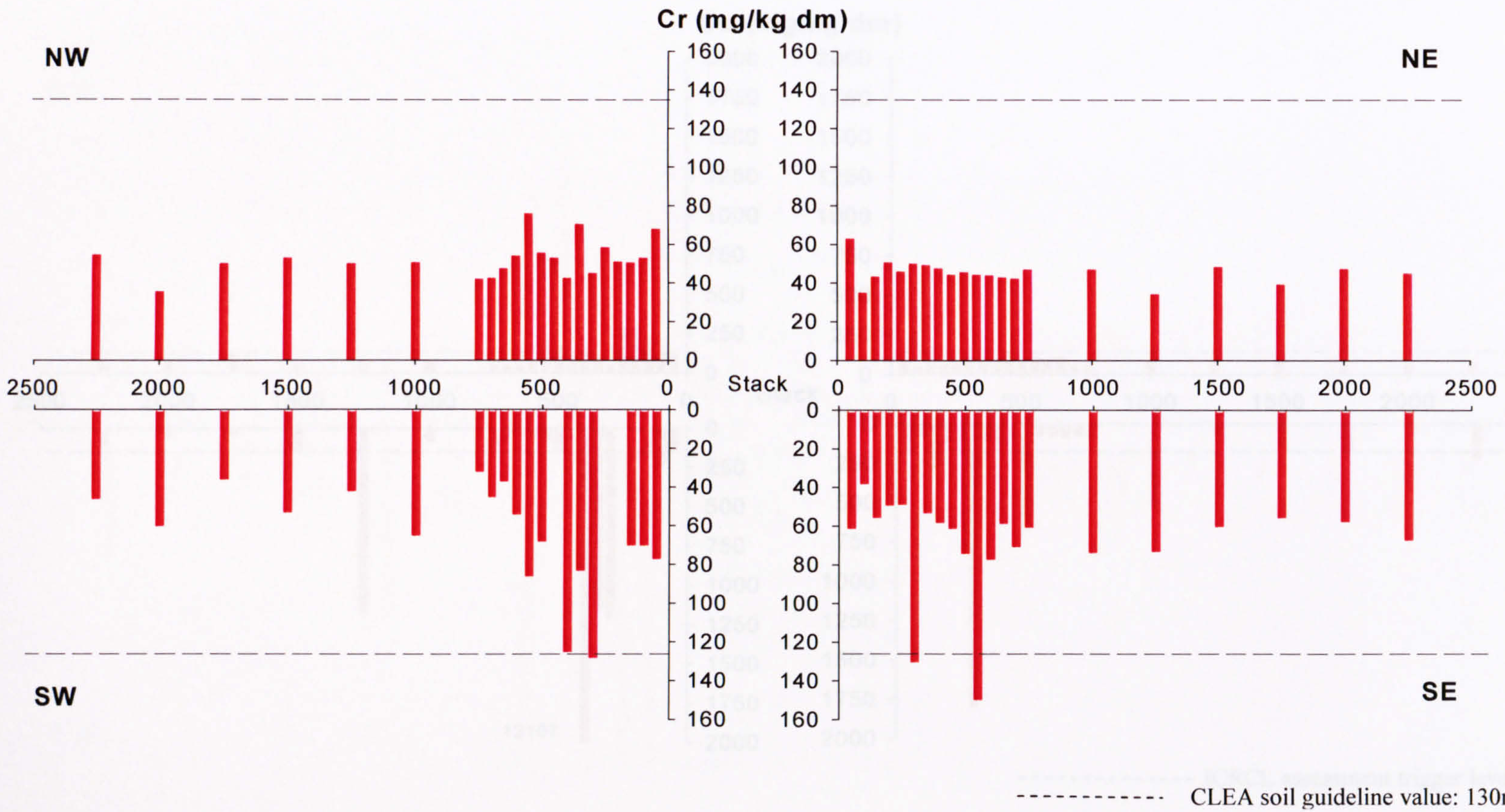


Figure 2.6 Mean soil chromium levels (mg/kg) by sector and distance band
NB mean values in NW, SE, NE sectors; single readings in SW sector. Adapted from Vizard et al (2003).

Copper

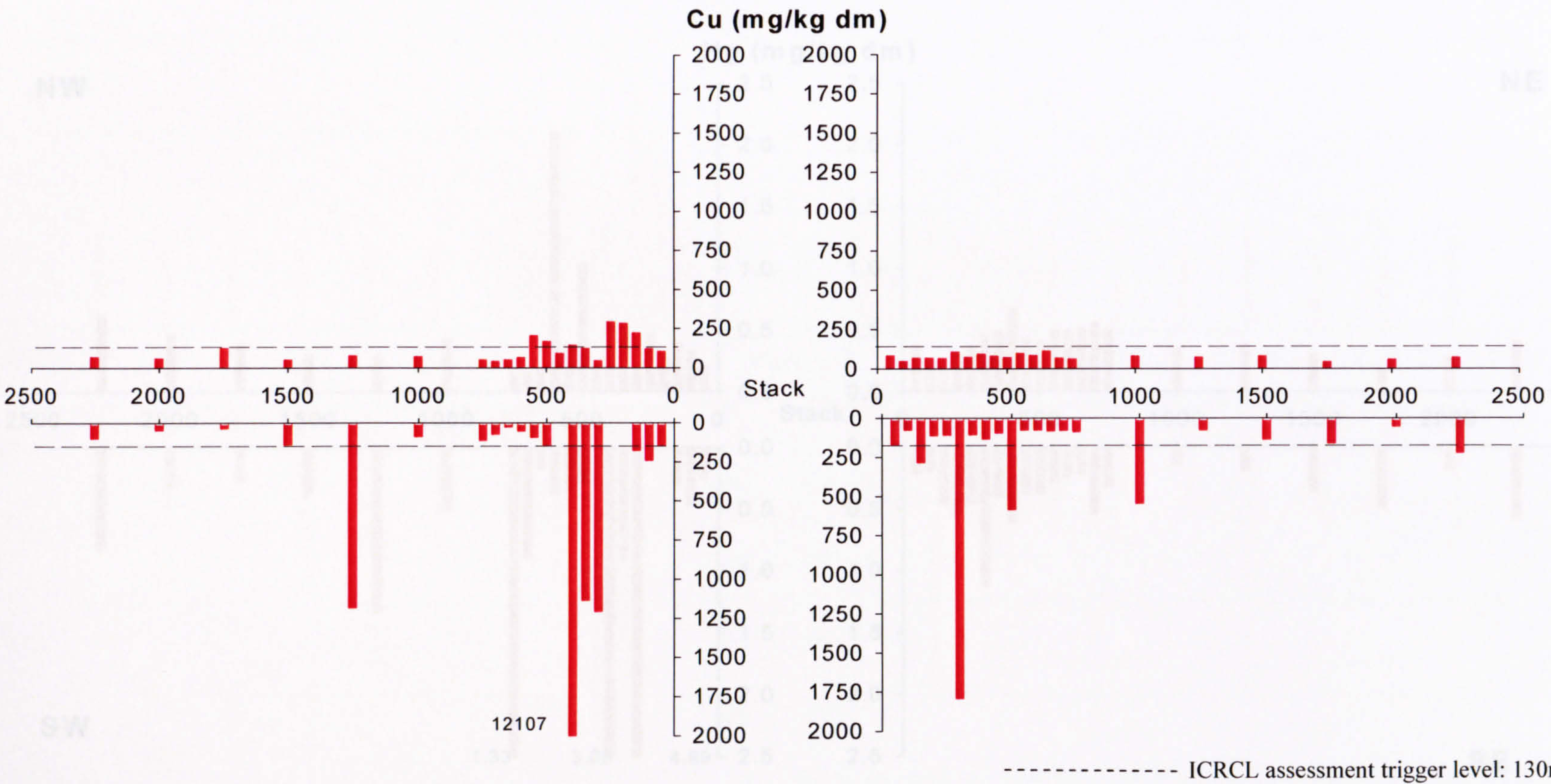


Figure 2.7 Mean soil copper (mg/kg) by sector and distance band NB mean values in NW, SE, NE sectors; single readings in SW sector. Adapted from Vizard et al (2003).

Mercury

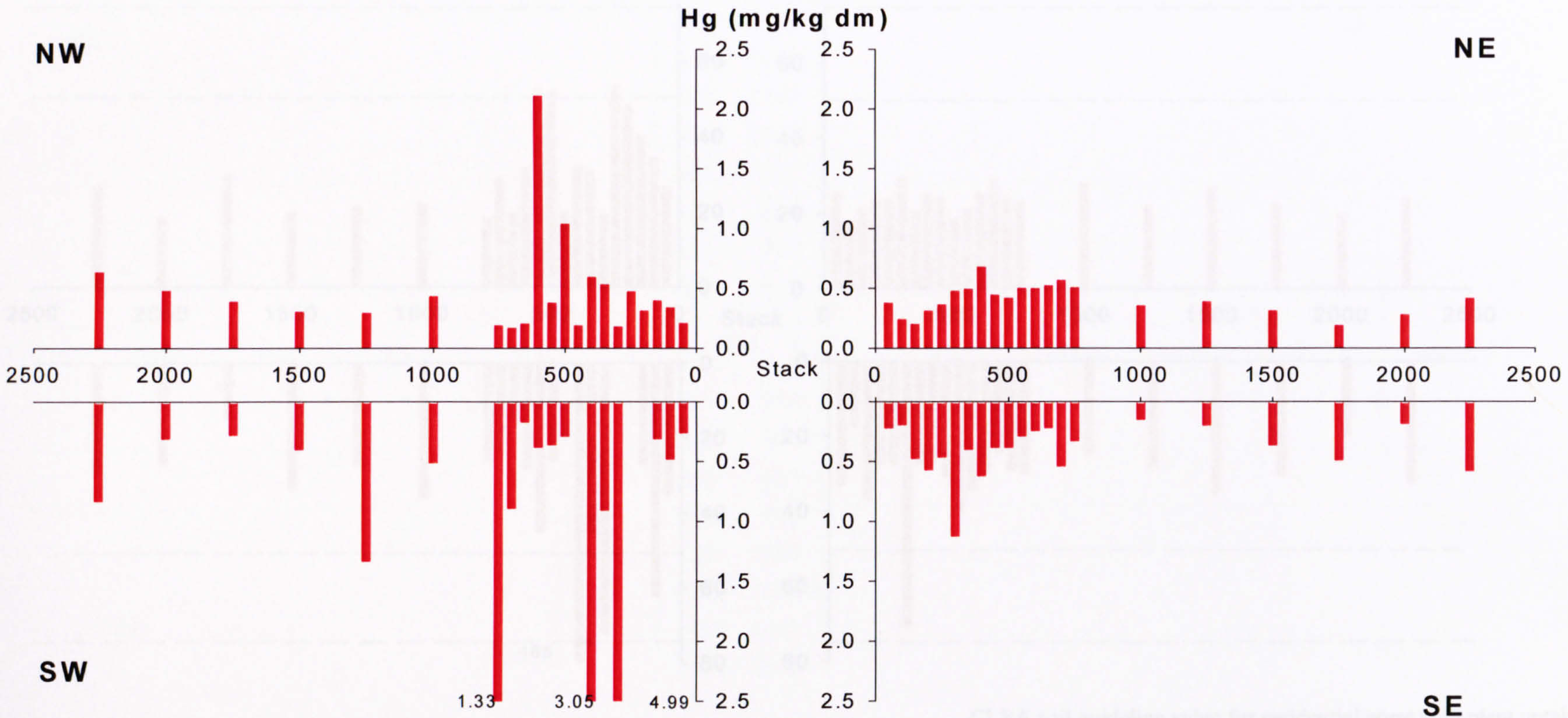


Figure 2.8 Mean soil mercury (mg/kg) by sector and distance band. Adapted from Vizard et al (2003).

Nickel

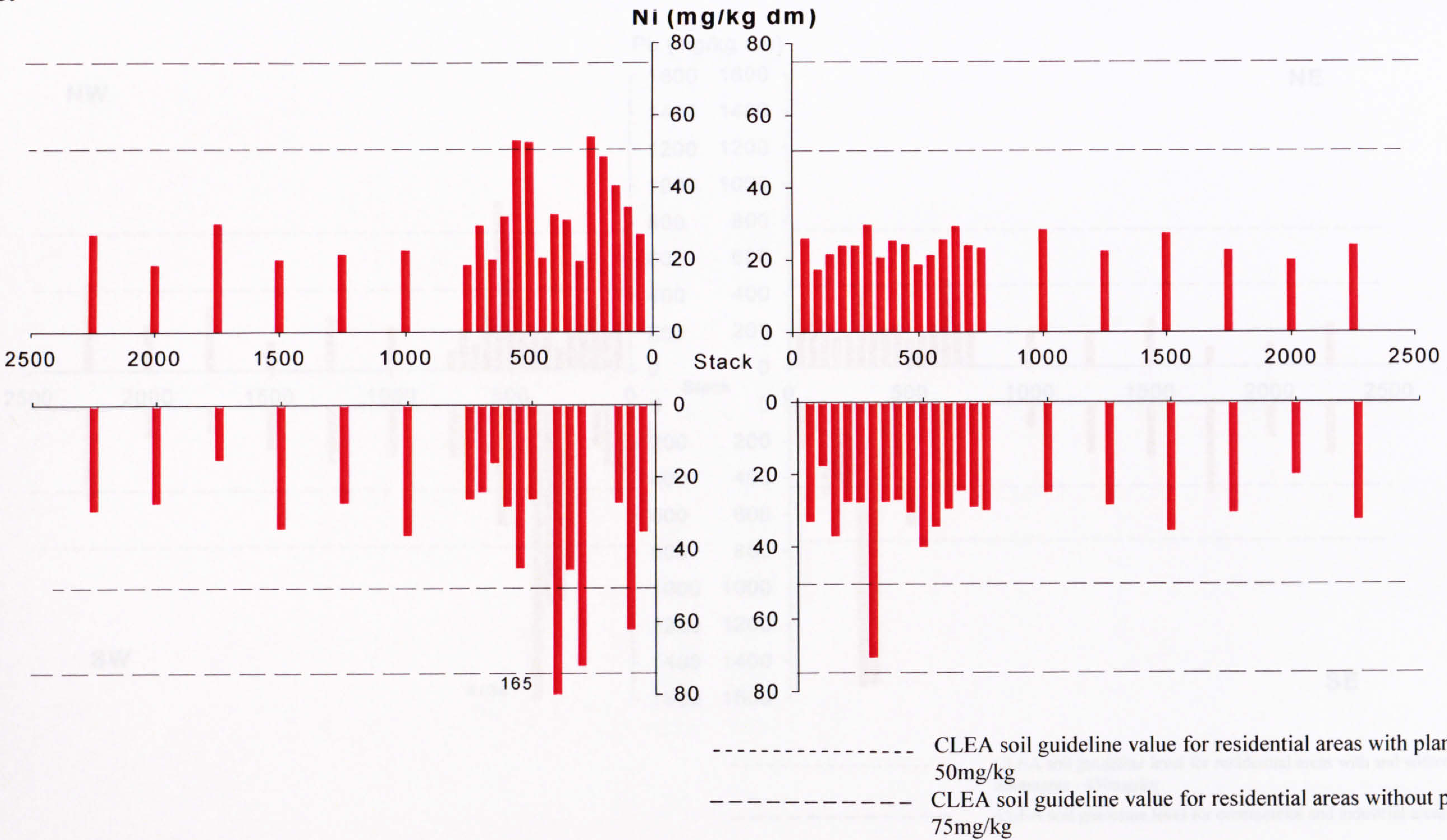


Figure 2.9 Mean soil nickel (mg/kg) by sector and distance band. Adapted from Vizard et al (2003).

Lead

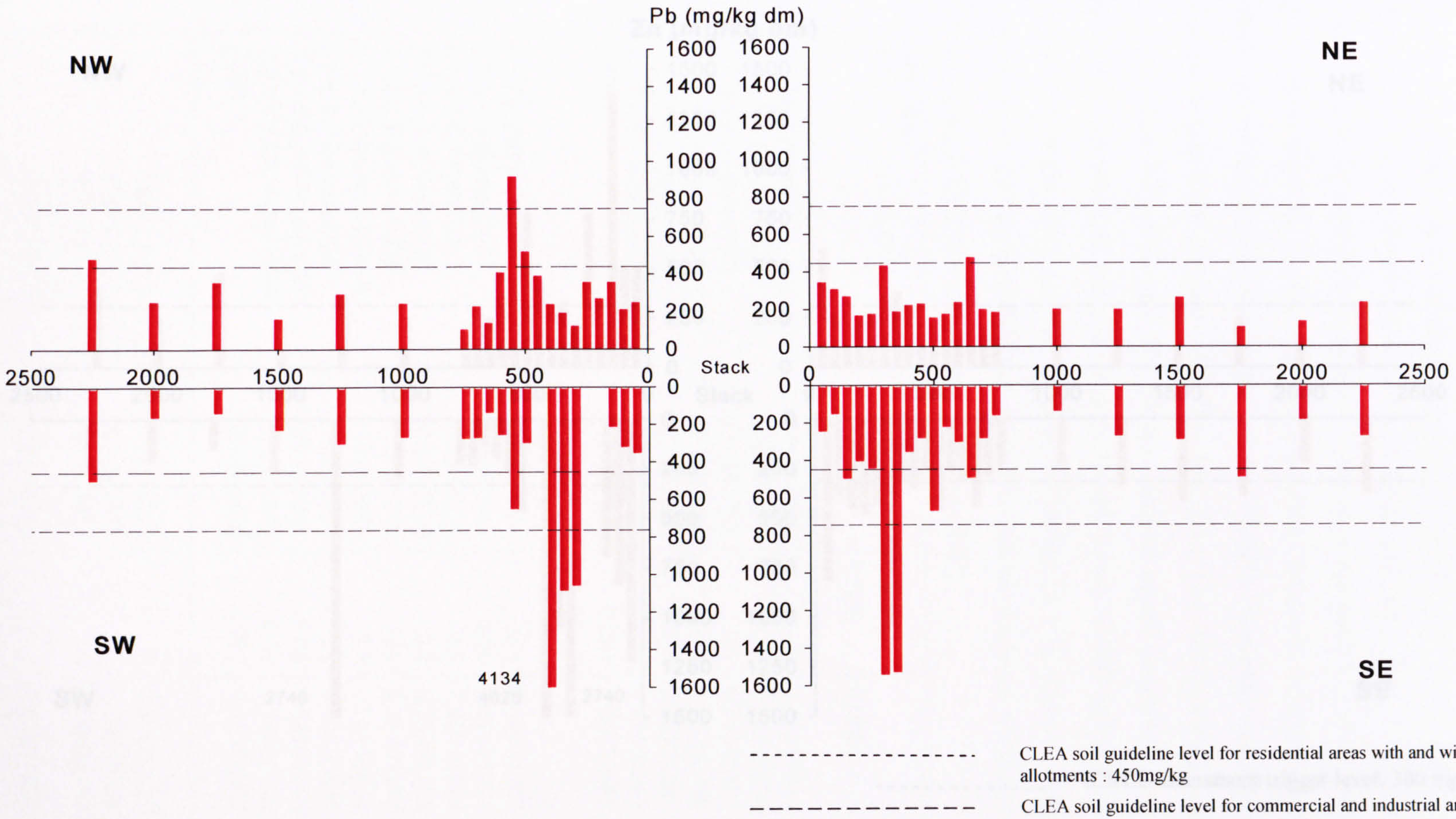


Figure 2.10 Mean soil lead levels (mg/kg) by distance band and sector. Adapted from Vizard et al (2003).

Zinc

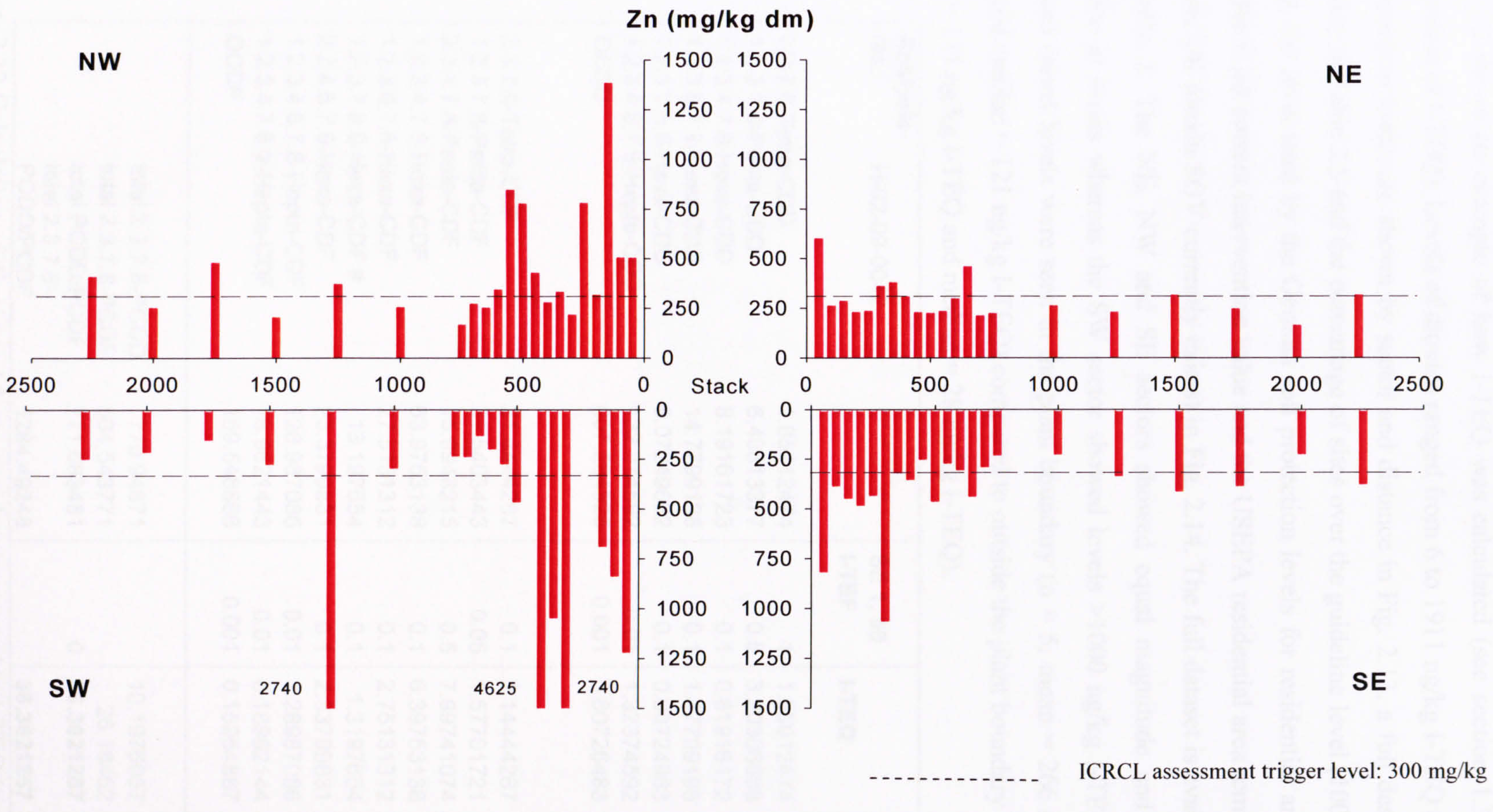


Figure 2.11 Mean soil zinc levels (mg/kg) by sector and distance band. Adapted from Vizard et al (2003).

2.3.2 Dioxins levels in soil samples

Fig. 2.12 shows an example of how I-TEQ was calculated (see section 1.3 for an explanation of I-TEQ). Levels of dioxins ranged from 6 to 1911 ng/kg I-TEQ (n = 82); concentration levels are shown by sector and distance in Fig. 2.13, a full descriptive summary in Table 2.5 and the percentage of sites over the guideline level (1000 ng/kg I-TEQ, the level used by the German soil protection levels for residential areas, the new Dutch list current intervention value and the USEPA residential area remediation goal- no UK dioxin SGV currently exists) in Fig. 2.14. The full dataset is available in Appendix 2. The NE, NW and SE sectors showed equal magnitude and similar variation in levels whereas the SW sector showed levels >1000 ng/kg I-TEQ. Also localised raised levels were seen in the plant boundary (n = 5, mean = 266 ng/kg I-TEQ and median = 121 ng/kg I-TEQ) compared to outside the plant boundary (n = 77, mean = 111 ng/kg I-TEQ and median = 28 ng/kg I-TEQ).

Analysis-No.: H-02-09-0042		**SE 1, 50 I-TEF**	**I-TEQ**
2.3.7.8-Tetra-CDD	1.85912474	1	1.85912474
1.2.3.7.8-Penta-CDD	6.40613377	0.5	3.20306688
1.2.3.4.7.8-Hexa-CDD	8.19161723	0.1	0.81916172
1.2.3.6.7.8-Hexa-CDD	14.7799198	0.1	1.47799198
1.2.3.7.8.9-Hexa-CDD	9.07249632	0.1	0.90724963
1.2.3.4.6.7.8-Hepta-CDD	132.374592	0.01	1.32374592
OCDD	607.264825	0.001	0.60726483
2.3.7.8-Tetra-CDF	21.4444267	0.1	2.14444267
1.2.3.7.8-Penta-CDF	31.5403443	0.05	1.57701721
2.3.4.7.8-Penta-CDF	15.9948215	0.5	7.99741074
1.2.3.4.7.8-Hexa-CDF	63.9753138	0.1	6.39753138
1.2.3.6.7.8-Hexa-CDF	27.5131312	0.1	2.75131312
1.2.3.7.8.9-Hexa-CDF #	13.197654	0.1	1.3197654
2.3.4.6.7.8-Hexa-CDF	23.3799831	0.1	2.33799831
1.2.3.4.6.7.8-Hepta-CDF	128.987086	0.01	1.28987086
1.2.3.4.7.8.9-Hepta-CDF	18.9621443	0.01	0.18962144
OCDF	159.548866	0.001	0.15954887
total 2.3.7.8-PCDD	779.94871		10.1976057
total 2.3.7.8-PCDF	504.543771		26.16452
total PCDD/PCDF	771.683451	0	**36.3621257**
total 2.3.7.8-PCDD/PCDF	1284.49248		**36.3621257**

Figure 2.12 Calculation of dioxin and furan raw data converted to I-TEQ in ng/kg; Sample SE1,50 shown.

Dioxins

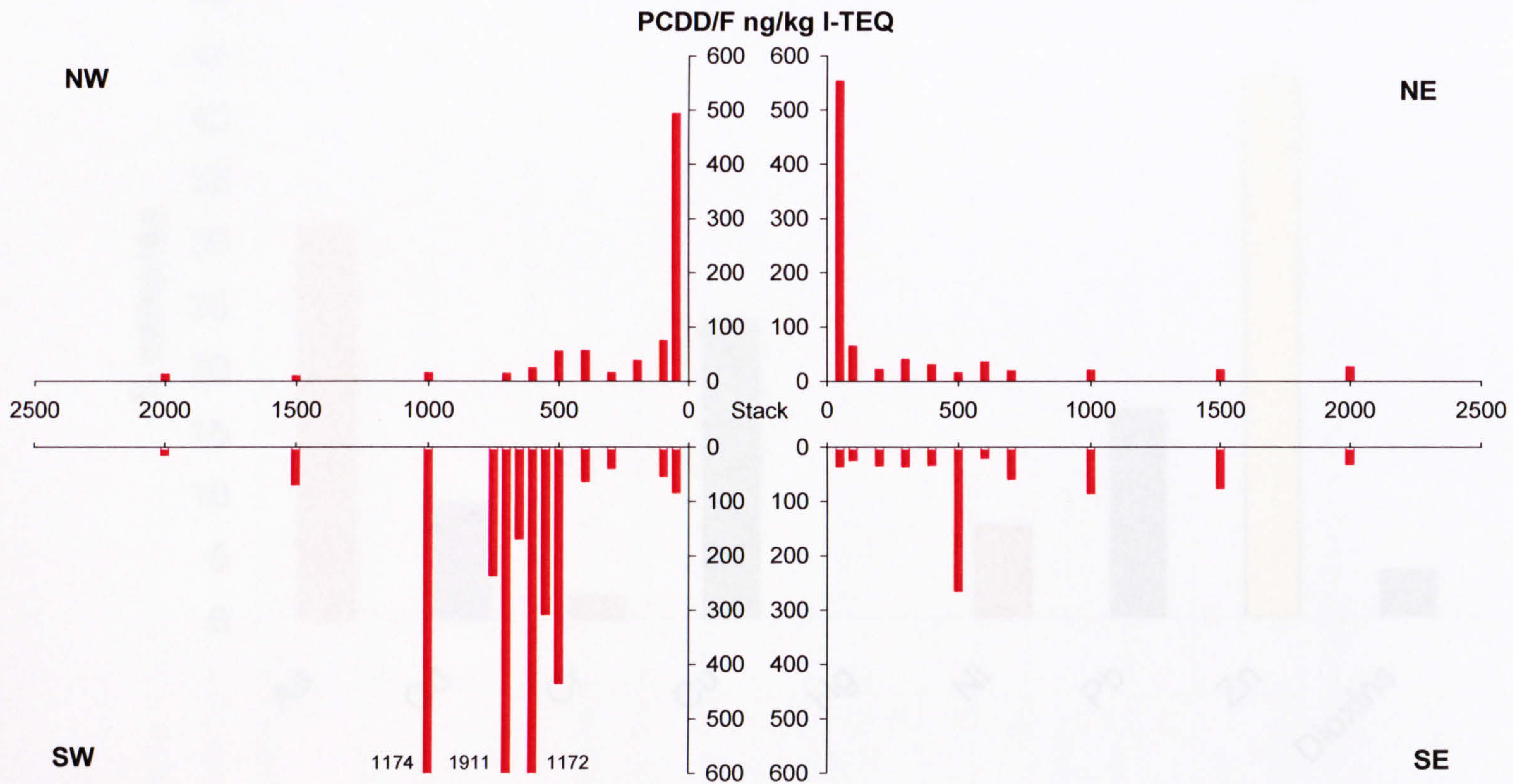


Figure 2.13 Soil dioxins (ng/kg I-TEQ) by distance band and sector NB mean values in NW, SE, NE sectors; single readings in SW sector. Adapted from Vizard et al (2003).

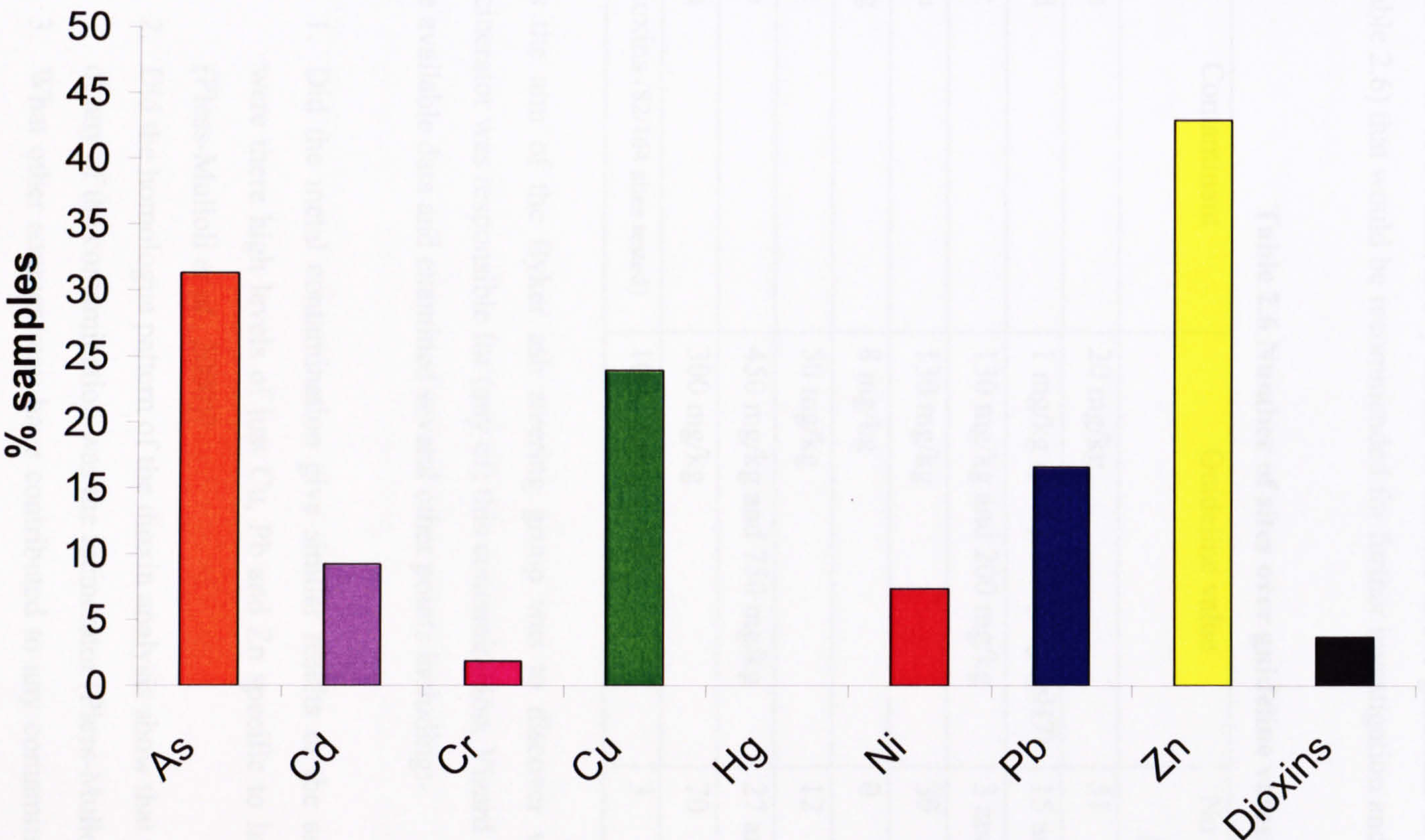


Figure 2.14 Percentage of sites with levels of arsenic, heavy metals and dioxins over guideline values (CLEA, ICRCL and various); As=20 mg/kg, Cd=1 mg/kg (pH6), 2 mg/kg (pH7) Cr=130 mg/kg and 200 mg/kg, Cu=130 mg/kg, Hg=8 mg/kg, Ni=50 mg/kg, Pb=450 mg/kg and 750 mg/kg, Zn=300 mg/kg and dioxins=1000 ng/kg I-TEQ

2.4 DISCUSSION

Assessment of 163 sites surrounding the incinerator site showed a total of 90 locations with levels of arsenic, heavy metals or dioxins above guideline or trigger values (see Table 2.6) that would be recommended for further investigation and/or remediation.

Table 2.6 Number of sites over guideline values

Contaminant	Guideline value	No of sites exceeding guideline value
As	20 mg/kg	51
Cd	1 mg/kg (pH6), 2 mg/kg (pH7)	15 and 7
Cr	130 mg/kg and 200 mg/kg	3 and 2
Cu	130 mg/kg	39
Hg	8 mg/kg	0
Ni	50 mg/kg	12
Pb	450 mg/kg and 750 mg/kg	27 and 13
Zn	300 mg/kg	70
Dioxins (82/164 sites tested)	1000 ng/kg I-TEQ	3

As the aim of the Byker ash steering group was to discover whether the Byker incinerator was responsible for (any of) this contamination, Vizard *et al.*, (2003), used the available data and examined several other points including:-

1. Did the metal contamination give similar results to the ash from Byker i.e. were there high levels of just Cu, Pb and Zn specific to incinerator samples, (Pless-Mulloli *et al.*, 2000)?
2. Did the homologue pattern of the dioxin analysis show that the primary source of any of the contamination was the incinerator (Pless-Mulloli *et al.*, 2000)?
3. What other sources may have contributed to any contamination (historic and current land use)?

The findings from Vizard *et al.*, (2003) and Pless-Mulloli *et al.*, (2000) are discussed here in conjunction with other available literature and with the specific aims of this thesis in mind (see section 2.1.4).

2.4.1 Historic and current use land survey

A combination of Ordnance Survey (OS) maps and currently available data was examined to see whether any other past or present industries were potentially responsible for, or may have contributed to, any contamination found. OS maps from 1856, 1898, 1921, 1936, 1950 and circa 1960-1980 were examined for the whole study area. Over 400 different industrial plants have existed in the study area between 1856 and 1980. These included industries associated with metal manufacture, dock yards and ship building, chemical works, glass and building material works, timber treatment, railway and engineering works and many others. Figure 2.15 (adapted from (Aspinall *et al.*, 1988) shows an illustration on the growth of industry on Tyneside over the past ~300 years. Metals may have been deposited by many of these endeavours whereas dioxins could only have been caused for a few processes.

For current sources of industry capable of contributing to soil contamination a current land use survey was carried out. The Pollution Inventory, kept by the Environment Agency (www.environment-agency.gov.uk) was examined for Part A processes - industries known to contribute substantial environmental pollution - up to 5 miles around the Byker incinerator site. Industries that pollute to a lesser extent – known as Part B processes – are currently licensed by the local authority and data was supplied by the relevant local councils.

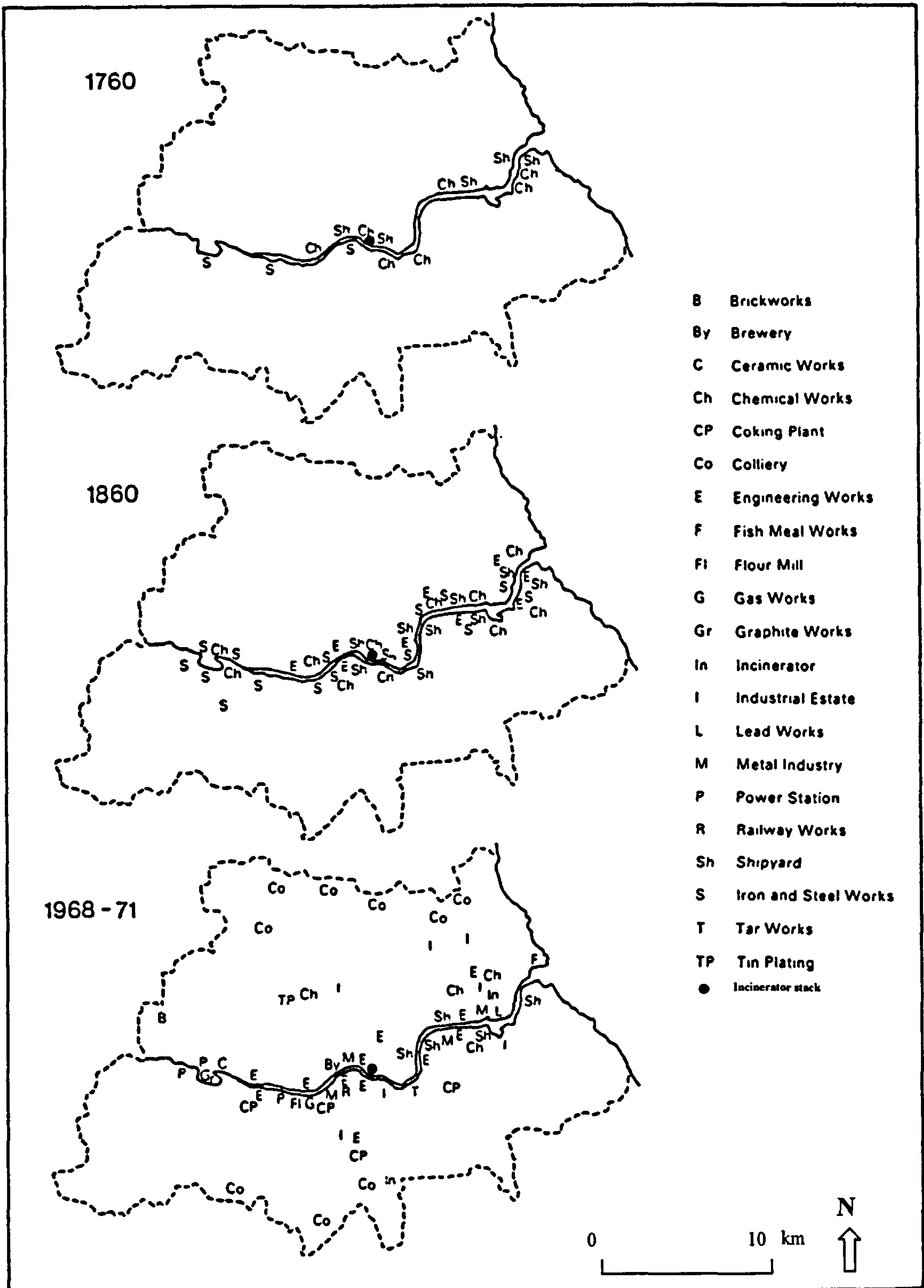


Figure 2.15 The distribution and range of industry on Tyneside between 1760 and 1971 - adapted from Aspinall *et al.*, (1988).

2.4.2 Arsenic and heavy metals levels in Tyneside soils

High levels of co-located Cu, Pb and Zn were found in the previous study into the influence of the ash on footpaths (Pless-Mulloli *et al.*, 2000). A correlation matrix displaying Spearman's rank correlation coefficient and p-values was used for arsenic and heavy metals on a sector basis. If just the 3 metals were found then contamination could be related to the ash and the incinerator. No evidence for joint elevation without the presence of other metals was found and in general, levels were correlated in all sectors (Vizard *et al.*, 2003).

The historic and current use land survey showed several potential sources of arsenic and heavy metal contamination either within, or close to the sampling area. Calder Industrial Materials Ltd produces up to 22,000 tonnes of lead per annum, Resinous Chemicals Ltd is known to emit copper, lead, nickel and zinc and Elementis UK Ltd, situated south of the Byker site, just outside the study area emits mercury. Part B processes that may have contributed to soil contamination include British Engineering Ltd (Foundry division) which manufactures iron, stainless steel, aluminium/alloy and copper alloy and may emit arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc. Until recently another foundry (Parson Power Generations Systems Ltd) to the north-north-east of the study area could also have contributed to metal contamination.

Several "hotspots" of contamination were found, mainly concentrated in the SW (although this sector had the lowest sampling density); if the incinerator was responsible for these elevated levels of arsenic and heavy metals then the results from the NE sector should have been consistently raised compared to the other sectors, this was not the case. Mean levels of As, Cd, Cr and Ni were similar or within range of UK background levels whereas Cu, Hg, Pb and Zn means were all elevated above background (see Tables 2.1 and 2.5 in this report or the same data shown in Rimmer *et al.*, 2005). Compared to other research performed on Newcastle soils, numerical data is only available to compare levels of Pb and Zn from the Wallsend Burn (Mellor, 2001) where levels found were much lower than those from this study (<50% of Byker means). So although concentrations tended to be elevated above background

levels and typical of urban areas, contamination specifically from the incinerator could not be detected.

2.4.3 Dioxin and Furan levels in Tyneside soils

Vizard *et al.*, (2003) went on to analyse the patterns of the dioxins and furans (the homologue distribution known as a "signature" or "fingerprint") as this method can be used to identify the source of the contamination. The study by Pless-Mulloli *et al.*, (2000) had discovered a characteristic zigzag shape (TCDD< PCDD< HxCDD< HpCDD< OCDD and HxCDF> HpCDF> OCDF) that was found in 14/16 Byker ash samples and was henceforth referred to as the "Byker pattern" (see Fig 2.16).

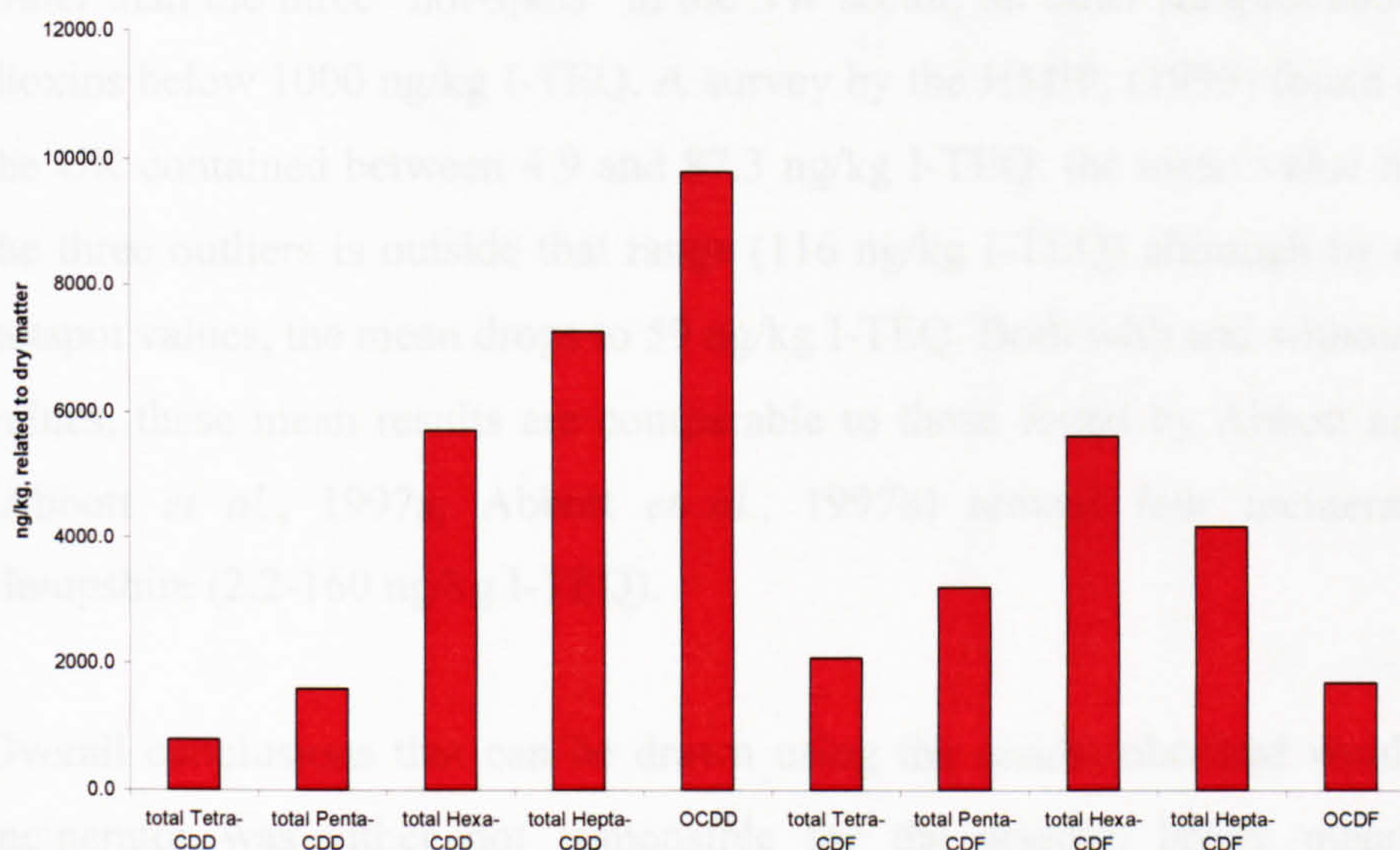


Figure 2.16 An example of the "Byker pattern" (sample shown is NE1, 50 adapted from Vizard et al 2003)

Of the 86 samples analysed, 4 main homologue patterns were identified; the Byker, deposition (bell-shaped), furan-dominated and OCDD-dominated patterns (shown in Fig 2.16). There were also several samples with unidentifiable patterns which may have formed due to a mixture of influences. The distribution of the homologue patterns across the study area is shown in Figure 2.18; it showed the Byker pattern (16/86 samples ~19%) was found at a maximum of 400 m away from the plant with the majority of samples within the plant boundary and all but 3 samples showed levels of under 100 ng/kg I-TEQ.

The extensive historical land use study showed a potential source of the highest levels of dioxin contamination in the SW sector, in the Saltmeadows area of Gateshead. The Allhusen's chlor-alkali plant dominated the area in the 19th century (Campbell, 1964); a furan-dominated homologue pattern of dioxins (see Fig 2.17) is a known by-product of the electrolytic process used on the site (Zook & Rappe, 1994) and is similar to the furan-dominated pattern actually found at Saltmeadows making the process the most likely source of the contamination found.

Of the Part A processes registered, two businesses were found that are known to emit dioxins (Calder Industrial Materials Ltd and Resinous Chemicals Ltd), just west-south-west outside the study area.

Other than the three "hot-spots" in the SW sector, all other samples showed levels of dioxins below 1000 ng/kg I-TEQ. A survey by the HMIP, (1995) found urban soils in the UK contained between 4.9 and 87.3 ng/kg I-TEQ; the mean value here including the three outliers is outside that range (116 ng/kg I-TEQ) although by excluding the hotspot values, the mean drops to 59 ng/kg I-TEQ. Both with and without the outlying values, these mean results are comparable to those found by Abbott and colleagues (Abbott *et al.*, 1997a; Abbott *et al.*, 1997b) around four incinerators in rural Hampshire (2.2-160 ng/kg I-TEQ).

Overall conclusions that can be drawn using the results obtained would be that the incinerator was either not responsible for the arsenic, heavy metal and dioxin contamination found and/or background contamination makes it difficult to see any contributions specifically made by the incinerator. It appears that past industrial actions are the main cause of contamination. The study has given a unique insight into the issue of soil contamination in urban Newcastle (and the wider Tyneside area) and it is likely that other UK urban areas and cities are equally contaminated.

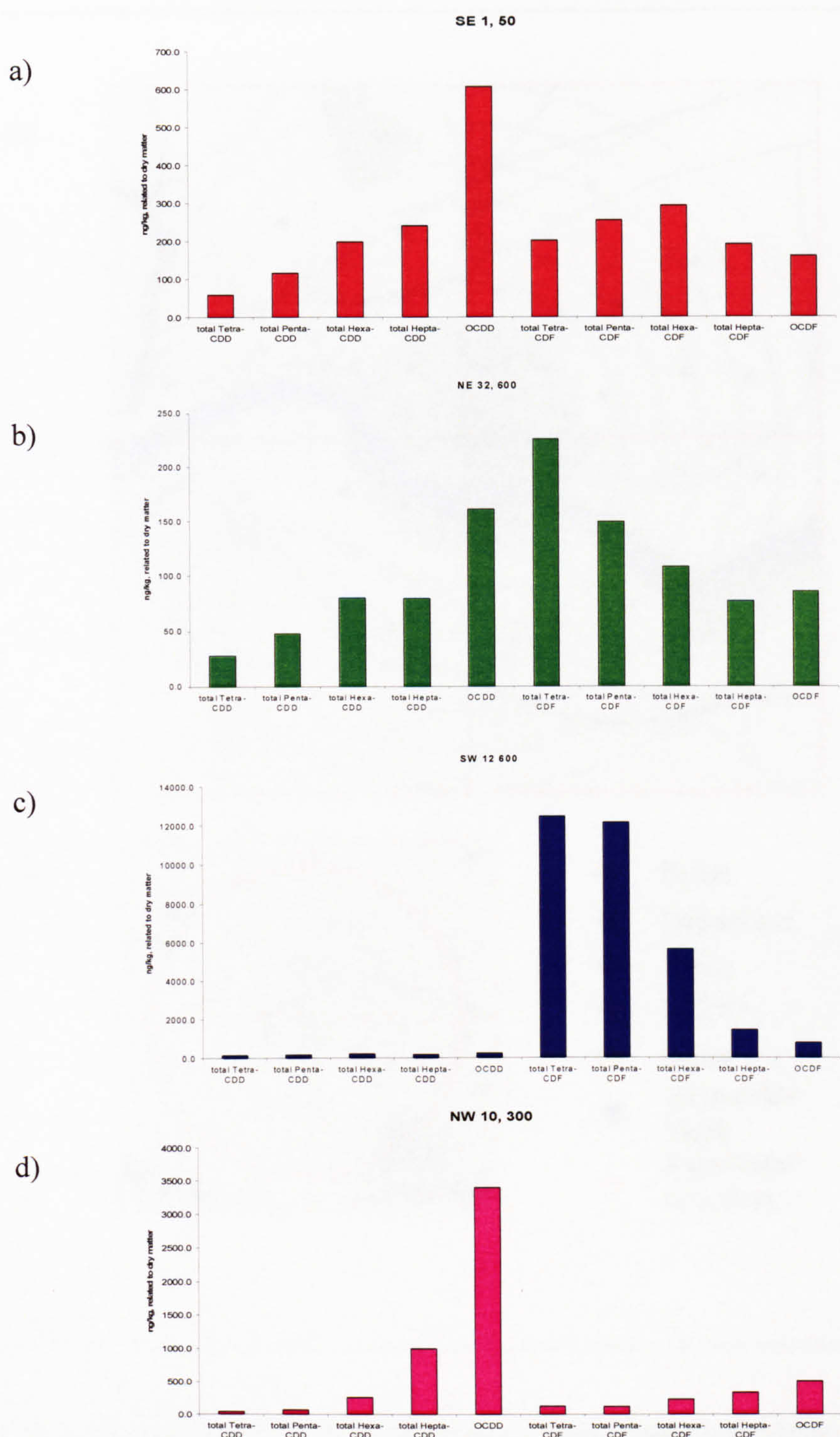


Figure 2.17 Homologue patterns a) Byker b) Deposition c) Furan dominated d) OCDD dominated.

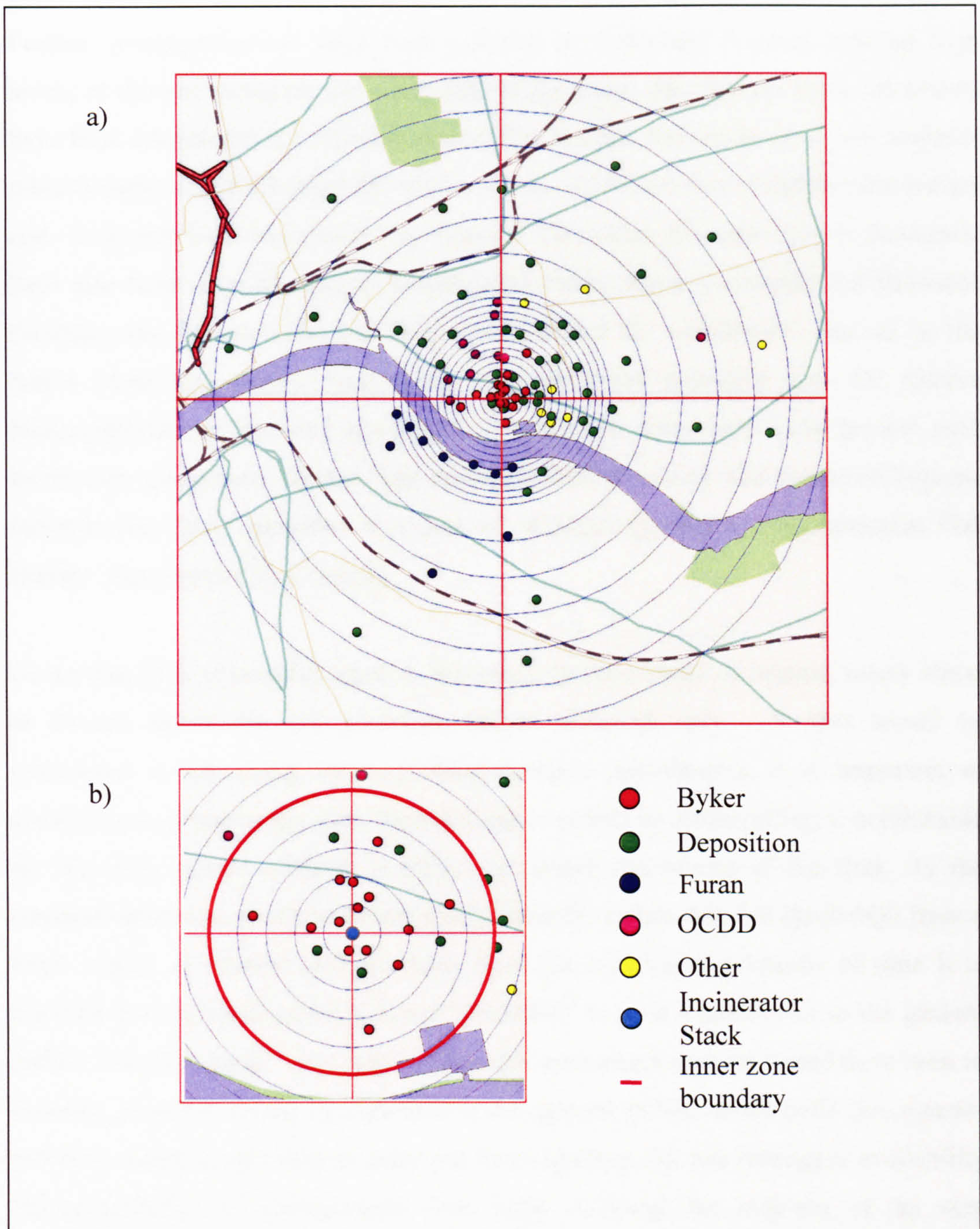


Figure 2.18 Distribution of dioxin homologue patterns a) across the study area and b) in the inner zone

2.4.4 Investigations and actions taken as a consequence of the studies

Further investigation has since been initiated by Gateshead Council into the high levels of dioxins found on the south side of the river. The Walker Road allotments have been remediated (i.e., the soil removed to landfill, the site levelled and facilities rebuilt) using a DEFRA grant of ~£1.8 million, ~£250,000 from English Partnerships and ~£100,000 from Newcastle City Council. Two other allotment sites in Newcastle have also been more thoroughly investigated, Nun's Moor (Gosforth) and Branxton (Walker); the Branxton site has been recommended for remediation whereas on the Nun's Moor plot, further assessment was performed including tests for relative bioaccessibility of lead and arsenic (the 2 pollutants discovered to be present with levels over guidelines). Rather than remediate, health advice, and improved hygiene facilities have been provided at a cost of ~£50,000 (personal communication Phil Hartley, Newcastle City Council).

Given that 55% of samples taken in this study showed levels of arsenic, heavy metal or dioxins above the soil guideline values (although only ~ 9 sites would be considered a risk using source-pathway-receptor assessment), it is important to consider a response to this fact. Remediating over half an urban setting is unthinkable so, the other option outlined in CLEA is further assessment of the sites. As the contamination was shown to be not solely from the incinerator, but (probably) from a huge variety of sources and has been there for long/varying lengths of time it is possible that the contamination is not "available" to pose a health risk to the general public. The most usual "next step" in any risk assessment process would have been to examine potential routes of exposure of the general public to the soils (i.e. source-pathway-receptor) and then to carry out investigations into the biological availability (bioaccessibility) of contaminants from soils. Although the majority of the sites sampled were public open spaces it is possible that in the future some may be redeveloped. This work was interested in considering bioavailability issues and methods, which as described in the paragraph above, can be used to more realistically assess risks to human health and are the subject of Chapter 3.

Chapter 3 - Investigation into the availability of heavy metals from urban Tyneside soils

3.1 INTRODUCTION

3.1.1 Availability of contaminants: comparison of several extraction techniques

The results generated by chemical analysis of soils in the Byker project (see Chapter 2 and Vizard *et al.*, 2003) gave specific information on the total levels of arsenic, heavy metals and dioxins present in urban soils surrounding the Byker incinerator. Ninety of the 163 samples showed levels of metal/dioxin over UK guideline values; assuming there are a source, pathway and receptor, the approach laid down in CLEA recommends further assessment, although the specific form of assessment required is not given. From the metal analysis results generated in Chapter 2, it is not possible to know whether the soils actually pose a risk to human or ecosystem health. It is now generally accepted that "total" amounts of a contaminant are unable to predict the likelihood of harm being caused (Alexander, 2000). Bio(logical)availability of contamination and actual toxicity to receptor organisms (humans, fish, bacteria etc) is more scientifically relevant and site specific assessment using this measure may have cost benefits (Ehlers & Luthy, 2003).

Many factors may affect the ability of a contaminant to be harmful including;-

- The original source (natural or industrial) or nature (solid ore or water soluble form etc) of contamination and its residence time in the soil (aging).
- Speciation of a contaminant and interaction between contaminants (potential antagonistic or synergistic effects e.g. An *et al.*, 2004)
- Basic physical soil factors e.g. pH, particle size of soil
- The receptor at risk (humans, crops, ecosystems, buildings)

Given that contaminated land guidance exists, in the main, to protect human health standards it is important to focus on the main routes that humans are exposed to soils. The primary routes are through inhalation, ingestion (of soils directly or leaching into drinking water) and dermal contact. Paustenbach's 2000 review of exposure assessment (Paustenbach, 2000) listed the oral/ingestion pathway as being of greater significance than dermal or respiratory exposures when assessing contaminated land risks. Directly feeding humans contaminated soil is unethical, expensive, time consuming and difficult to reproduce; therefore, investigations mimicking what happens to soil when it is ingested are key to understanding how contamination that enters the human body may potentially cause harm. To date only Maddaloni *et al.*, (1998) have carried out a human/contaminated soil feeding experiment; they fed Pb contaminated soil (2924 mg/kg) to 6 adult volunteers. With fasting, doses of 250 μgPb/70 kg body weight showed 26.2% absorption whereas with food only 2.52% was absorbed.

The term "bioavailability" in this thesis is defined as the fraction of the administered dose that reaches the blood stream (either through the gastrointestinal tract, lungs or skin) whereas the fraction that is soluble in the GI tract and available for absorption is referred to as bioaccessible (Paustenbach, 2000).

3.1.2 Oral bioaccessibility and bioavailability of soil contaminants

Investigations into the oral bioavailability of contaminants from soil have been pioneered by Michael Ruby and colleagues since the early 1980's (e.g. (Ruby *et al.*, 1993; 1996; 1999). Their focus has been on designing an *in vitro* extraction test for use in estimating oral bioavailability of soil contaminants (mainly of lead and arsenic) by validation against *in vivo* animal studies (e.g. (Casteel *et al.*, 1997; Freeman *et al.*, 1993; 1994; 1995; 1996), in order to reduce the need for further animal studies (which are expensive, time consuming and ethically problematic). *In vitro* extraction methods are able to provide information rapidly and inexpensively in order to characterize risk.

Due to the international recognition of the importance of bioavailability (and particularly the need to understand oral bioavailability) a parallel group of *in vitro* gastrointestinal analogue bioavailability tests have been developed in different countries. The Environment Agency (2002a) report reviewed the range of experimental protocols developed. The main variations between techniques (summarised in Table 3.1) include which gastrointestinal compartments are mimicked (i.e. mouth, stomach, small intestine and colon), residence time in each compartment, solid to liquid ratio (i.e. soil to body fluids) and pH and chemical composition of replica body fluids. The variations tend to be due to who the main receptor of the contaminated soil is thought to be – a small child who has not consumed food (fasting) represents the worst case scenario; inclusion of food has been shown to reduce the availability of lead (James *et al.*, 1985; Maddaloni *et al.*, 1998).

Not all of the techniques have been validated against *in vivo* models; a practical handbook and summary of bioavailability of metals from soils and *in vivo* methods was produced by Kelley *et al.*, (2002).

Table 3.1 Summary of the main features and variations of current gastrointestinal analogue extraction tests

Method name	Compartments	pH	Liquid/Solid ratio	Residence time (in hrs)	Analysis	Metals tested	Validation status
***	**						
PBET	2 3	2.5 7	100/1	1 4	solution solution x2	As, Pb	Swine, monkey (As, Pb)
SBET	2	1.5	100/1	1	solution	As, Cd, Pb	Swine (Pb)
IVG§	2 3	1.8 5.5	150/1		solution	As	Swine (As)
US P	2	ca.1	1000/1	2	solution	As, Cd, Cr, Ni .Pb	Not validated
MB & SR	1 2 3	ca.1 ca.1 ca.1	160/1 2160/1 4770/1	5 secs 2 4	solution and solid	As, Cd, Cr, Pb	Compared to humans (Pb)
DIN§	1 2 3	6.4 2 7.5	15/1 50/1 100/1	0.5 2 6	solution	As, Cd, Cr, Hg, Pb	Swine (unpublished)
SHIME§	2 3	5.2 6.5	2.5/1 4/1	3 5	solution	As, Cd, Pb	Compared
RIVM	1 2 3	6.5 1.1 5.5	15/1 37.5/1 97.5/1	5 mins 2 2	solution and solid	As, Cd, Pb	Compared
TIM *	1 2 3	5 2 7.2	5/1 30/1 51/1	5 mins 1.5 6	solution	As, Cd, Pb	Compared
AOAC	2	1.1 2	150/1	16	solution	Al, Cu, Fe, Mn, Zn	Not validated

adapted from Environment Agency, 2002a.

NB all methods were carried out at 37°C.

* indicates a flow-though method: all others were batch methods.

§ indicates that food was included in these methods: all other methods, no food included.

** 1=oral cavity, 2=stomach, 3=small intestine.

*** PBET – Physiologically Based Extraction Test, SBET - Simplified Bioaccessibility Extraction Test, IVG – *In-Vitro* Gastrointestinal Method, USP – US Pharmacopoeia Method, MB &SR – Mass Balance & Soil Recapture method, DIN – German DIN 00 19738, SHIME – Simulator of Human Intestinal Microbial Ecosystem of Infants, RIVM – *in vitro* Digestion Model, TIM – TNO Gastrointestinal Model, AOAC – Association of Analytical Communities Pepsin Digestibility Test.

3.1.3 – Selected methods for the assessment of the availability of soil contaminants

Water

Drinking water is also orally ingested and the risk of contamination of a water supply must also be assessed. Chemical extraction tests which mimic the leaching of metals and other contaminants from soils either by rainwater or landfill leachate were developed in order to assess any risks to (drinking) water supplies. For example, deionised water is used by both Lewin *et al.* (1994) and the ASTM (1999) D3987-85 to mimic leaching from soil; the Australian standard (Australian standard, 1997) uses deionised water, a high pH tetraborate solution and local water and the USEPA has a range of tests to simulate leaching by acid rain or landfill conditions (USEPA methods 1311, 1312, 1310A and 1320, USEPA, 2000).

***In vitro* physiological analogues**

Only one other study has examined the bioavailability of metals to human body fluids (other than gastrointestinal and water); Wainman *et al.*, (1994) extracted chromium (VI) into synthetic sweat and found available levels to be significantly lower than total extraction methods. Other approximations of human physiological fluids are available (for example growth media used for culturing human cells) and may provide an idea of how available contaminants are to cause harm once across the gastrointestinal barrier.

3.1.4 – Bioaccessibility and bioavailability of contaminants from urban Tyneside soils

Due to the prohibitive cost of dioxin analysis (> £400/sample), this work focused on heavy metal contamination. The results in Chap. 2 showed that, of the heavy metals (and As) analysed for, only four elements (Cu, Ni, Pb and Zn) were found to have levels that ranged widely (with samples above and below SGV's). A sub-set of 16 samples (all also characterised for dioxins) was selected, four with high levels of metal contamination, four with medium levels, four with low levels ("control" samples) and four with low levels of metal but high levels of dioxin. Figure 3.1 and Table 3.2 show the levels of Cu, Ni, Pb and Zn (dioxin levels also listed in the table) in the 16 samples.

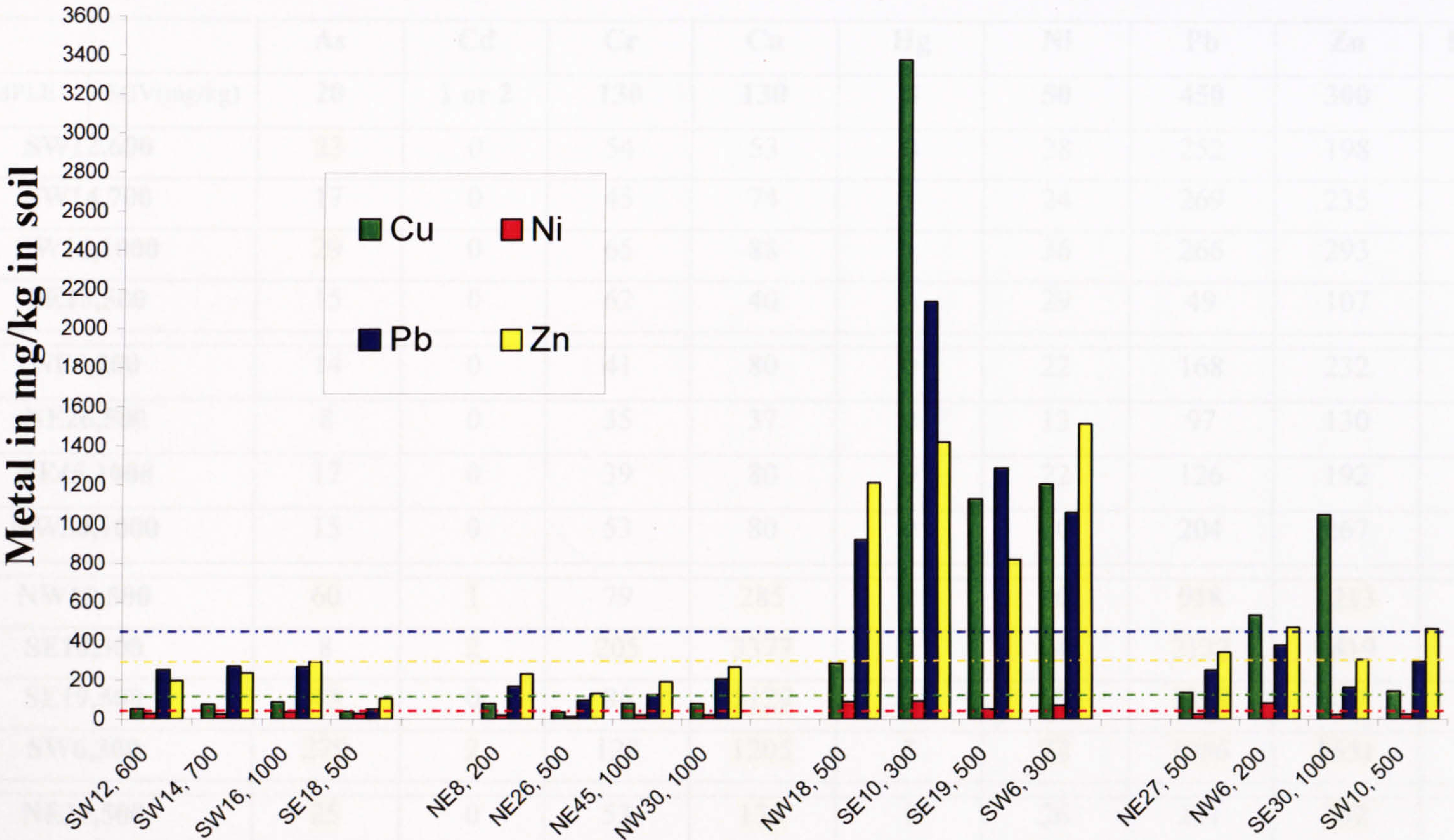


Figure 3.1 Levels of "total" Cu (green), Ni (red), Pb (blue) and Zn (yellow) in mg/kg in the subset of 16 samples from the Byker project. Dotted lines represent the soil guideline values, above which a sample requires further assessment; green=Cu, 130 mg/kg; red = Ni, 50 mg/kg; blue=Pb, 450 mg/kg; yellow=Zn, 300 mg/kg. Numerical values are given in Table 3.1

Table 3.2 Samples selected from Chapter 2 results for further analysis

SAMPLE	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn	Dioxins in ng/kg I-TEQ
SGV(mg/kg)	**20**	**1 or 2**	**130**	**130**	**8**	**50**	**450**	**300**	**1000**
SW12,600	23	0	54	53	0	28	252	198	1772
SW14,700	17	0	45	74	1	24	269	235	1911
SW16,1000	29	0	65	88	1	36	266	293	1174
SE18,500	15	0	62	40	0	29	49	107	481
NE8,200	14	0	41	80	0	22	168	232	19
NE26,500	8	0	35	37	0	13	97	130	9
NE45,1000	17	0	39	80	0	22	126	192	13
NW30,1000	15	0	53	80	0	20	204	267	16
NW18,500	60	1	79	285	2	90	918	1213	102
SE10,300	8	2	205	3377	2	94	2137	1419	19
SE19,500	43	0	86	1129	1	51	1287	817	49
SW6,300	279	2	128	1205	5	72	1056	1551	39
NE27,500	25	0	53	135	1	26	251	342	25
NW6,200	49	0	60	532	0	82	377	470	66
SE30,1000	13	0	71	1046	0	23	161	306	154
SW10,500	34	2	68	144	0	26	296	463	435

Highlighted in yellow are over guideline values, CLEA or ICRCL

3.1.5 Experimental aims

Assuming that all soils present a risk (based on the source-pathway-receptor model), this work sought to find out what percentage of the contaminated soils would present a risk to humans. This work specifically investigated the bioaccessibility and bioavailability (using selected techniques) of copper, nickel, lead and zinc from 16 soils (eight metal contaminated, eight not) sampled from urban Tyneside (levels of total dioxin and metal contamination pre-characterised) in order to further establish whether these contaminated soils were a risk to humans.

A range of soil extraction techniques were used in order to:-

1. Assess the availability of Cu, Ni, Pb and Zn from the soils to water
2. Assess the availability of the four metals from the soils using two physiological simulated gut protocols (mimicking the human ingestion process) and compare the methods
3. Assess the availability of the four metals from the soils to a physiological human fluid (human tissue culture medium)

A further aim was to investigate whether extractability of the four metals from the soils could be related to the physical properties of soils - pH, levels of organic carbon and particle size.

3.2 MATERIALS AND METHODS

3.2.1 Soil/Water extract

This method was adapted from the NRA (National Rivers Authority) leaching test of Lewin *et al.*, (1994). 1 g of soil was weighed into a 50 ml plastic centrifuge tube. 10 ml of dH_2O was added and the tube shaken for 5 secs by hand. The mixture was then shaken at 100 rpm (orbital shaker, Sanyo Gallenkamp) for 1 hr at 27°C. To remove particulate matter and microbial contamination the solutions were filtered (0.2 µm polypropylene, Whatman) and stored at 4°C in the dark until metal analysis. As a control a sample without soil was included as a blank. All samples and blanks were performed in triplicate. This method was performed to simulate levels of metal available to plants and water supplies.

3.2.2 Soil/Simulated gut extracts

Several different gastrointestinal analogue methods exist (see Table 3.1); 2 different methods (RIVM and BGS PBET) were carried out in order to discover if the variations in the methods produced substantial differences in the results obtained and for comparative purposes to discover which method was technically easier to carry out.

RIVM method

The method used was adapted from that described in Sips *et al.*, (2001). All glass wear was acid washed and rinsed with deionised water before use. 4 main solutions were made up: Saliva, Pancreatic, Duodenal and Bile.

Saliva solution

Inorganic 10 ml KCl (89.6 g/L), 10 ml KSCN (10 g/500 ml), 10 ml NaH_2PO_4 (88.8 g/L), 10 ml Na_2SO_4 (28.5 g/500 ml), 1.7 ml NaCl (360.6 g/2 L) and 1.8 ml NaOH (2 g/50 ml) were added to a 500 ml volumetric flask and made up to 500 ml with dH_2O.
Organic 8 ml urea solution (25 g/L) was made up to 500 ml with dH_2O.
The inorganic and organic solutions were mixed together using a magnetic stirrer. 145 mg α-amylase, 15 mg uric acid and 50 mg mucin were dissolved into the solution.
The required pH of the final solution was pH 6.5. Any deviation from this was corrected with NaOH or HCl as appropriate

Pancreatic Solution

Inorganic 15.7 ml NaCl (350.6 g/2 L), 3 ml NaH_2PO_4 (88.8 g/L), 9.2 ml KCl (89.6 g/L), 18 ml $CaCl_2.2H_2O$ (2.22 g/100 ml), 10 ml NH_4Cl (1.53 g/50 ml) and 8.3 ml 37% HCl (8 ml HCl in 992 ml deionised H_2O) were made up to 500 ml with dH_2O.

Organic 10 ml Glucose solution (65 g/L), 10 ml Glucuronic acid solution (2 g/L), 3.4 ml urea solution (25 g/L) and 10 ml Glucoseamine hydrochloride solution (33 g/L) were made up to 500 ml with dH_2O.

The inorganic and organic solutions were mixed using magnetic stirring. 1 g bovine serum albumin (BSA), 1 g Pepsin and 3 g Mucin were dissolved into the solution. The required final pH of this solution was pH 1.07; any deviation was corrected with NaOH or HCl as appropriate.

Duodenal Solution

Inorganic 80 ml NaCl (350.6 g/2 L), 80 ml $NaHCO_3$ (169.4 g/2 L), 20 ml KH_2PO_4 (8 g/L), 12.6 ml KCl (89.6 g/L), 20 ml MgCl (5 g/L) and 360 µl 37%HCl were made up to 500 ml using dH_2O.

Organic 8 ml urea solution (25 g/L) was made up to 500 ml with dH_2O.

The inorganic and organic solutions were mixed with a magnetic stirrer. 18 ml $CaCl_2.2H_2O$ (2.22 g/100 ml), 2 g BSA, 6 g Pancreatin and 1 g Lipase were added and dissolved into the solution. The required final pH was pH 7.8; any deviation was corrected with NaOH or HCl as appropriate.

Bile Solution

Inorganic 30 ml NaCl (350.6 g/2 L), 68.3 ml $NaHCO_3$ (169.4 g/2 L), 4.2 ml KCl (89.6 g/L) and 200 µl 37% HCl were made up to 500 ml using dH_2O.

Organic 10 ml urea solution (25 g/L) was made up to 500 ml with dH_2O.

The inorganic and organic solutions were mixed with a magnetic stirrer. 10 ml $CaCl_2.2H_2O$ (2.22 g/100 ml), 1.8 g BSA and 6 g Bile were dissolved into solution. The final pH required was pH 8.0; any deviation was corrected with NaOH or HCl as appropriate.

All solutions were warmed to 37°C in a water bath; samples were also maintained at 37°C in an orbital shaker (Sanyo Gallenkamp) with gentle mixing at 100 rpm. As a control measure, a blank without soil was also created. All samples were carried out in triplicate.

Experimental Method

0.5 g of soil sample was weighed into a 50 ml centrifuge tube. 7.5 ml of saliva solution was added to the soil and incubated for 5 mins. 11.25 ml of pancreatic solution was added to the soil and saliva solution and incubated for 2 hrs. Finally, 22.5 ml of duodenal solution and 7.5 ml bile solution were added and the whole solution was incubated for a further 2 hrs. The tubes were then centrifuged for 5mins at 2750 g and this final supernatant (chyme) was retained and stored at 4°C in the dark until analysed for metal content. This measurement was designed to simulate the amount of metal bioaccessible in the small intestine.

BGS PBET method

The method described was taken from Cave et al (2002) and carried out during a visit to the BGS (British Geological Survey) labs, Keyworth, Nottingham.

6.25 g pepsin, 2.5 g sodium citrate, 2.5 g sodium malate, 2.1 ml lactic acid and 2.5 ml glacial acetic acid were made up to 5 L with deionised H_2O and the pH was adjusted to pH 2.5 with conc. HCl. The solution was referred to as "simulated gastric solution".

1 g of soil sample was weighed into a HDPE (high density polyethylene) bottle and 100 ml of simulated gastric solution added. This was rotated end-over-end (30 rpm) in a 37°C water bath for 1 hr.

5 ml of solution was removed and passed through a 0.45 μm cellulose acetate disk filter. This filtered sub-sample was labelled "stomach" (Stom). 5 ml clean gastric fluid was then back-flushed through the filter into each sample bottle in order to maintain the solid:solution ratio. The pH was altered to 7 using saturated sodium bicarbonate. 0.175 g bile salts and 0.05 g pancreatin were dissolved into each sample.

The samples were then rotated for 2 hrs in the 37°C water bath. 5 ml of fluid was extracted and labelled "small intestine 1" (Int1). The samples were rotated for a further 2 hrs and a 5 ml sample taken at the end of this time was labelled "small intestine 2" (Int2) i.e. 3 different measurements (Stom, Int1 and Int2) were taken. Samples were stored at 4°C in the dark until analysed for metal content.

The capacity of the water bath was 20 samples, allowing in this instance for 1 blank, 16 samples, a repeat of one sample, and a standard reference material. The reference material used was the NIST (National Institute of Standards and Technology) Standard Reference Material 2710, a Montana soil with highly elevated trace element concentrations..

3.2.3 Soil/DMEM extract

As with the soil/water extract method (section 3.2.2), 1 g of soil was shaken with 10 ml of DMEM (a human cell culture medium – Gibco) for 1 hr at 37°C at 100 rpm (orbital shaker – Sanyo Gallenkamp). To remove particulate matter and microbial contamination the solutions were filtered (0.2 μm polypropylene, Whatman) and stored at 4°C in the dark until metal analysis. As a control a "soil-less" sample was included as a blank; all samples were performed in triplicate. This method was performed in order to determine biosolubility, similar to Langley-Turnbaugh et al (2005).

3.2.4 Metal analysis

All samples were kept at 4°C in the dark for a maximum of two days before analysis.

AAS

An ILS 357 Atomic Absorption Spectrometer (Instrumental Laboratory Systems 357) was used to analyse the water, RIVM simulated gut and DMEM extracts. Metal standards used to calibrate the machine were all Spectrosol R (BDH) 1000 mg/L solutions diluted to 0.5, 1, 1.5, 2, 3 and 4 mg/L for Cu, Pb and Ni and 0.1, 0.2, 0.4, 0.6, 0.8 for Zn. Samples exceeding the calibration range were diluted (usually two or five fold) until within range. All samples were measured in triplicate.

ICP-AES

Extracts generated by the BGS PBET method were analysed for metal content at the BGS labs, Keyworth, Nottingham using ICP-AES with a Varian Vista axially viewed instrument. Quality control included blank standards, sample repeats and standard reference material standards. The instrument was calibrated using six mixed metals standards, (made up in the same liquid matrix as samples) with concentrations ranging from 0.05 mg/L to 50 mg/L. The instrument was re-calibrated every 20 samples with standards 1-6; two quality control standards (low and high range) were also run every 10 samples. All reported measurements were based on the mean of three 10 sec replicate analyses.

3.2.5 Selected soil physical and chemical properties

Soil particle size

The method used was adapted from Rowell (1994) by Dr R.S.Shiel (personal communication) following Stokes Law of streamlined flow:

25 g soil was added to 5 ml of Calgon (10% solution) and 200 ml H_2O. This was shaken for 4 hrs, left to stand over night and then shaken for a further 4 hrs. The suspension was passed through a 212 μm sieve (course sand fraction) over a 63 μm sieve (fine sand fraction) and the effluent collected. The sieves/sand were dried at 35°C over night and the residue then weighed. The effluent was poured in a 1 L measuring cylinder, made up to 1 L with dH_2O and thoroughly agitated. A sample was immediately taken at 10 cm depth using an Andreason pipette (20 ml) and added to a clean pre-weighed 100 ml beaker (silt + clay fraction). The sample was re-agitated and left for 4 hrs. A sample was then taken at a depth of 5 cm using the Andreason pipette (20 ml) and added to a clean pre-weighed 100 ml beaker (clay only fraction). The beakers were left until all the water had evaporated and then weighed.

In order to calculate the percentage of sand/silt/clay the weights taken at 10 cm and 5 cm (minus beaker weight) were multiplied by 50 (20 ml from 1 L= 1/50th), 0.6 g was taken away (Calgon weight) and the "clay only" reading was taken from the "silt + clay" reading. The combined weights then added up to the original 25 g and % was calculated.

pH

As described in Rowell (1994), 10 g soil was added to 25 ml dH_2O. The suspension was shaken for 15 mins and then left to settle for 30 secs. At 20°C, pH was measured using a Jenway PHM6 and a glass slurry electrode, calibrated using colour key buffers (BDH) at pH 4, 7 and 10.

Total Organic Carbon

An adaptation of the Walkley-Black method (Black, 1965) for carbon and organic matter was used. 1 g soil was added to 10 ml potassium dichromate and 20 ml conc. sulphuric acid, swirled for 1 min and left to stand for 30 mins. 200 ml dH_2O, 10 ml conc. phosphoric acid and 2 ml (barium) diphenylamine sulphonate indicator was then added. This solution was then titrated against ferrous sulphate (ferrous Fe II sulphate 0.5 M). The end point was a colour change from dark green/blue to pale green. Results were calculated using the following formula and expressed as a percentage:-

TOC = (((blank titre in ml-sample titre in ml) * 0.3 * 0.5M)/weight of soil in g)*1.3

Cation Exchange Capacity

Cation exchange capacity (CEC) was calculated using the method of Helling *et al.* (1964) - which used pH and the percentage of clay and organic matter to calculate CEC.

CEC (in cmol/kg) = ((clay%/100) * clay factor) + ((OM%/100) * OM factor)

NB – for pH 6, clay factor = 56 and OM factor = 131, for pH 7, clay factor = 60 and OM factor = 163.

3.2.6 – Statistical analysis

MINITAB® 14 Statistical software for Windows was used to perform regression analysis of the metal and basic soil parameter data in order to investigate which, if any, parameter most influenced bioavailability.

3.3 RESULTS

The levels of metals extracted from the four different methods are expressed in three different but complimentary ways here:-

- As mg of metal per kg soil (mg/kg) in the same way as results were expressed for "total" metals for all the Byker soils in Chapter 1
- As a % extraction; i.e. what percentage of the total metal has been extracted using a particular technique?
- As mg of metal per L of extraction fluid used. This is in order to know how much metal is present in liquid preparations that may be used further in subsequent biological tests.

NB – it must be noted that directly comparing SGV values to levels of metal extracted using bioaccessibility methods is not strictly valid. However, any bioaccessible level found to be exceeding the SGV will, undoubtedly, have a total level of metal above the SGV.

3.3.1 – Soil/Water extract

Table 3.3 shows the amounts of Cu, Ni, Pb and Zn extracted from the 16 Byker soils selected, using the water extraction method. Only one sample (SE18, 500 for Pb) showed extraction efficiency >1% whilst the highest actual levels of extractable metal were 2.19 mg/kg Cu (SW6,300), 0.3 mg/kg Ni (SE18,500), 0.64 mg/kg Pb (SE18,500) and 3.44 mg/kg Zn (NW18,500). The low extraction efficiencies would indicate that most of the metals are not or only sparingly water soluble (or at least not soluble at pH 7 – physiological pH).

Table 3.3 Soil/water extracts

Sample name	Cu in mg/kg		%	Cu	Ni in mg/kg		%	Ni	Pb in mg/kg		%	Pb	Zn in mg/kg		%	Zn
	Total	H_2O		mg/l	Total	H_2O		mg/l	Total	H_2O		mg/l	Total	H_2O		mg/l
SW12, 600	53	0.22	0.42	0.02	28	n.d.	0	n.d.	252	n.d.	0	n.d.	198	0.29	0.14	0.03
SW14, 700	74	0.45	0.61	0.05	24	0.03	0.12	0.00	269	0.06	0.02	0.01	235	0.08	0.03	0.01
SW16, 1000	88	0.53	0.60	0.05	36	n.d.	0	n.d.	266	0.17	0.06	0.02	293	0.57	0.20	0.06
SE18, 500	40	0.10	0.24	0.01	29	0.30	1.03	0.03	49	0.64	1.29	0.06	107	0.18	0.17	0.02
NE8, 200	80	0.75	0.94	0.07	22	n.d.	0	n.d.	168	0.24	0.14	0.02	232	0.51	0.22	0.05
NE26, 500	37	0.19	0.51	0.02	13	n.d.	0	n.d.	97	0.06	0.06	0.01	130	0.31	0.24	0.03
NE45, 1000	80	0.37	0.47	0.04	22	n.d.	0	n.d.	126	0.12	0.09	0.01	192	0.18	0.09	0.02
NW30, 1000	80	0.46	0.57	0.05	20	n.d.	0	n.d.	204	0.00	0.00	0.00	267	0.22	0.08	0.02
NW18, 500	285	1.06	0.37	0.11	90	n.d.	0	n.d.	918	n.d.	0	n.d.	1213	3.44	0.28	0.34
SE10, 300	3377	0.08	0.00	0.01	94	0.09	0.10	0.01	2137	n.d.	0	n.d.	1419	0.00	0.00	0.00
SE19, 500	1129	1.43	0.13	0.14	51	n.d.	0	n.d.	1287	0.06	0.00	0.01	817	0.71	0.09	0.07
SW6, 300	1205	2.19	0.18	0.22	72	n.d.	0	n.d.	1056	0.00	0.00	0.00	1511	1.01	0.07	0.10
NE27, 500	135	0.51	0.38	0.05	26	n.d.	0	n.d.	251	n.d.	0	n.d.	342	1.28	0.37	0.13
NW6, 200	532	0.71	0.13	0.07	82	0.06	0.07	0.01	377	0.23	0.06	0.02	470	0.27	0.06	0.03
SE30, 1000	1046	0.95	0.09	0.10	23	n.d.	0	n.d.	161	0.11	0.07	0.01	306	0.25	0.08	0.03
SW10, 500	144	0.85	0.59	0.08	26	n.d.	0	n.d.	296	0.48	0.16	0.05	463	0.60	0.13	0.06

Total and extractable amounts of Cu, Ni, Pb and Zn shown in mg/kg, % extraction efficiency and mg/L. Highlighted in yellow are samples over the guideline values – Cu, 130 mg/kg; Ni 50 mg/kg; Pb 450 mg/kg; Zn 300 mg/kg. n=3. n.d. = not detected

3.3.2.1 Soil/simulated gut; RIVM extract

Table 3.4 shows amounts of Cu, Ni, Pb and Zn that were extracted from the 16 selected Byker soils using the RIVM simulated gut method. Extraction efficiency was much higher and more varied than results obtained using the water extraction method.

Cu extractability ranged from 0.62% (SE10,300) to ~ 30% (SW6,300) with one sample showing an extraction value in excess of the guideline value (>130 mg/kg) , sample SW6,300 (372.34 mg/kg). Ni demonstrated consistently low extractability (0-6.5% range) with the highest value being 5.33 mg/kg (from sample NW6,200). Pb showed even lower extractability with all levels <1% available (highest value – 10.08 mg/kg from SW6,300). Extractability of Zn was more varied than Ni and Pb (but less than Cu), ranging from 0~7.5% with the highest level being 74.41 mg/kg (NW18,500).

As well as giving high extractability (372.34 mg Cu/kg) using the RIVM method, sample SW6,300 also exhibited the highest actual amount of Cu that was extractable using the soil/water extract (2.10mg Cu/kg). The samples showing the highest levels of extractable Ni (NW6,200) and Pb (SW6,300) were not the same as the soil/water extract (sample SE18,500). Sample NW18,500 exhibited the highest level of extractable Zn using both the water and RIVM methods. The greater range of extractability using the RIVM method would suggest that the changes in pH during the extraction procedure release the metals from the soil.

Table 3.4 Soil/simulated gut (RIVM method) extracts.

Sample name	Cu in mg/kg		%	Cu	Ni in mg/kg		%	Ni	Pb in mg/kg		%	Pb	Zn in mg/kg		%	Zn
	Total	RIVM		mg/l	Total	RIVM		mg/l	Total	RIVM		mg/l	Total	RIVM		mg/l
SW12, 600	53	6.21	11.8	0.06	28	n.d.	0	0.00	252	n.d.	0	n.d.	198	5.81	2.93	0.06
SW14, 700	74	8.50	11.56	0.09	24	n.d.	0	n.d.	269	n.d.	0	n.d.	235	1.73	0.73	0.02
SW16, 1000	88	6.46	7.32	0.07	36	0.01	0.02	0.00	266	n.d.	0	n.d.	293	n.d.	0	n.d.
SE18, 500	40	4.01	9.99	0.05	29	n.d.	0	n.d.	49	n.d.	0	n.d.	107	n.d.	0	n.d.
NE8, 200	80	22.34	27.93	0.25	22	n.d.	0	n.d.	168	n.d.	0	n.d.	232	4.50	1.94	0.05
NE26, 500	37	2.52	6.76	0.03	13	n.d.	0	n.d.	97	n.d.	0	n.d.	130	n.d.	0	0.00
NE45, 1000	80	13.50	16.92	0.14	22	n.d.	0	n.d.	126	n.d.	0	n.d.	192	2.69	1.40	0.03
NW30, 1000	80	16.28	20.26	0.17	20	n.d.	0	n.d.	204	n.d.	0	n.d.	267	13.00	4.86	0.14
NW18, 500	285	32.07	11.27	0.32	90	n.d.	0	0.00	918	3.16	0.34	0.04	1213	74.41	6.14	0.77
SE10, 300	3377	20.99	0.62	0.24	94	2.77	2.96	0.03	2137	n.d.	0	n.d.	1419	n.d.	0	n.d.
SE19, 500	1129	86.49	7.66	0.92	51	n.d.	0	n.d.	1287	0.82	0.06	0.01	817	31.42	3.85	0.34
SW6, 300	1205	372.34	30.91	4.24	72	3.74	5.22	0.04	1056	10.08	0.95	0.12	1511	53.28	3.53	0.61
NE27, 500	135	21.10	15.6	0.24	26	1.62	6.22	0.02	251	n.d.	0	n.d.	342	23.06	6.73	0.26
NW6, 200	532	119.67	22.5	1.17	82	5.33	6.47	0.05	377	n.d.	0	n.d.	470	24.94	5.31	0.24
SE30, 1000	1046	31.47	3.01	0.34	23	1.48	6.46	0.02	161	n.d.	0	n.d.	306	15.29	5.00	0.17
SW10, 500	144	19.26	13.3	0.20	26	n.d.	0	0.00	296	n.d.	0	n.d.	463	34.20	7.39	0.36

Total and extractable amounts of Cu, Ni, Pb and Zn shown in mg/kg, % extraction efficiency and mg/L. Highlighted in yellow are samples over the guideline values – Cu, 130 mg/kg; Ni 50 mg/kg; Pb 450 mg/kg; Zn 300 mg/kg. n=3 n.d. = not detected

3.3.2.2 – Soil/simulated gut; BGS extract

Copper

Extractable levels of Cu (from 16 Byker soils) using the BGS simulated gut method (Stom, Int1 and Int2 fractions) are presented in Table 3.5. Levels of Cu extracted into the Stom fraction ranged from 0.18~30% with sample SW6,300 (similar to the RIVM extract) exhibiting the highest % extraction and actual value in mg/kg (352.71 mg Cu/kg; over the 130 mg/kg guideline value). The Int1 and Int2 fractions showed very similar results to each other (0~35% range) with samples SW6,300 and SE19,500 results both being over the 130mg Cu/kg guideline value.

Nickel

Results for Ni are shown in Table 3.6 with the Stom fraction extractable levels ranging from 4-11%; Int1 and 2 were again similar to each other (range 3-8.5%) with all samples (in mg/kg) being less extractable than in the Stom fraction.

Lead

Extractable levels of Pb are summarised in Table 3.7; the Stom fraction ranged from 0~9% extractable with sample SW6,300 demonstrating the highest % extraction and actual value (91.13mg/kg). Int1 extractability ranged from 0~5% with all mg/kg values slightly lower than the Stom fraction. Int2 results were similar to Int1 although all were slightly lower.

Zinc

Results for extractable Zn are shown in Table 3.8. The Stom fraction exhibited a wide range of extractability (13~50%); 2 samples (NW18,500 and SW6,300) had levels over the guideline value of 300mg/kg. Levels of extractable Zn in Int1 ranged from 0-23% and all values were lower than the Stom fraction; SW6,300 still showed an extractable level above the guideline value (348.07 mg/kg). Int2 extractable values were all lower than Int1, with sample SW6,300 extractable levels dropping below the guideline value.

The availability of Cu rose with the rise in pH (from Stom to Int1 to Int2) and the pattern of the results was similar to the RIVM method. Sample SW6,300 again showed the highest level of extractability with SE19,500 showing the next highest (unlike RIVM but similar to water). Ni was more available at the lower pH and showed a greater range of extraction efficiency at all pH's using the BGS method than the RIVM and water methods. With Pb, the rise in pH coincided with a drop in extractability. Levels of extractable Zn dropped as the pH rose. Unlike the RIVM and water methods the highest levels of Zn were not seen from NW18,500, in this case the highest extractable levels were found from SW6,300.

Table 3.5 Soil/simulated gut (BGS method) extracts; Cu.

Sample name	Cu	Stomach			Int1			Int2		
	Total	mg/kg	%	mg/L	mg/kg	%	mg/L	mg/kg	%	mg/L
SW12, 600	53	4.58	8.71	0.05	15.93	30.28	0.14	17.11	32.52	0.16
SW14, 700	74	6.10	8.30	0.06	22.90	31.13	0.20	23.64	32.15	0.21
SW16, 1000	88	4.75	5.38	0.05	20.90	23.69	0.19	22.53	25.54	0.21
SE18, 500	40	2.51	6.26	0.03	6.11	15.24	0.06	6.60	16.45	0.06
NE8, 200	80	8.40	10.50	0.08	25.35	31.71	0.21	27.49	34.38	0.24
NE26, 500	37	2.17	5.82	0.02	8.85	23.74	0.08	9.47	25.41	0.09
NE45, 1000	80	6.99	8.76	0.07	23.52	29.47	0.21	24.88	31.18	0.23
NW30, 1000	80	5.75	7.15	0.06	22.02	27.39	0.20	22.95	28.55	0.22
NW18, 500	285	12.35	4.34	0.12	42.96	15.09	0.35	47.64	16.74	0.41
SE10, 300	3377	6.08	0.18	0.07	14.57	0.43	0.14	15.39	0.46	0.16
SE19, 500	1129	105.87	9.38	1.04	160.35	14.21	1.36	165.20	14.64	1.46
SW6, 300	1205	352.71	29.28	3.75	415.38	34.48	4.09	415.78	34.51	4.19
NE27, 500	135	16.11	11.91	0.15	42.19	31.20	0.33	45.01	33.28	0.37
NW6, 200	532	51.97	9.77	0.60	82.33	15.48	0.85	83.33	15.67	0.90
SE30, 1000	1046	13.19	1.26	0.15	43.21	4.13	0.41	48.12	4.60	0.47
SW10, 500	144	11.39	7.92	0.11	38.16	26.53	0.32	42.35	29.45	0.37

Total and extractable amounts of Cu shown in mg/kg, % extraction efficiency and mg/L.
Highlighted in yellow are samples over the guideline values – Cu, 130 mg/kg.

Table 3.6 Soil/simulated gut (BGS method) extracts; Ni.

Sample name	Ni	Stomach			Int1			Int2		
	Total	mg/kg	%	mg/L	mg/kg	%	mg/L	mg/kg	%	mg/L
SW12, 600	28	2.00	7.15	0.02	1.88	6.71	0.02	1.86	6.66	0.02
SW14, 700	24	1.58	6.69	0.02	1.53	6.47	0.01	1.54	6.55	0.01
SW16, 1000	36	1.88	5.21	0.02	1.83	5.08	0.02	1.90	5.25	0.02
SE18, 500	29	1.43	4.93	0.02	1.22	4.18	0.01	1.21	4.15	0.01
NE8, 200	22	1.86	8.49	0.02	1.76	8.05	0.01	1.85	8.45	0.02
NE26, 500	13	0.95	7.47	0.01	0.74	5.87	0.01	0.97	7.65	0.01
NE45, 1000	22	1.80	8.33	0.02	1.54	7.13	0.01	1.54	7.11	0.01
NW30, 1000	20	1.57	7.74	0.02	1.29	6.36	0.01	1.37	6.74	0.01
NW18, 500	90	3.71	4.12	0.04	3.31	3.68	0.03	3.64	4.04	0.03
SE10, 300	94	3.87	4.13	0.04	3.76	4.01	0.04	3.84	4.10	0.04
SE19, 500	51	2.24	4.42	0.02	1.56	3.09	0.01	1.75	3.46	0.02
SW6, 300	72	6.90	9.62	0.07	5.63	7.85	0.05	5.27	7.34	0.05
NE27, 500	26	2.87	11.01	0.03	2.09	8.02	0.02	2.22	8.52	0.02
NW6, 200	82	3.40	4.14	0.04	3.05	3.71	0.03	3.12	3.79	0.03
SE30, 1000	23	1.94	8.49	0.02	1.91	8.34	0.02	1.96	8.56	0.02
SW10, 500	26	1.75	6.63	0.02	1.40	5.30	0.01	1.60	6.03	0.01

Total and extractable amounts of Ni shown in mg/kg, % extraction efficiency and mg/L.
Highlighted in yellow are samples over the guideline values –Ni 50 mg/kg.

Table 3.7 Soil/simulated gut (BGS method) extracts; Pb.

Sample name	Pb	Stomach			Int1			Int2		
	Total	mg/kg	%	mg/L	mg/kg	%	mg/L	mg/kg	%	mg/L
SW12, 600	252	14.04	5.57	0.14	8.72	3.46	0.08	7.02	2.79	0.07
SW14, 700	269	19.21	7.13	0.19	8.92	3.31	0.08	7.45	2.76	0.07
SW16, 1000	266	11.20	4.21	0.11	5.59	2.10	0.05	4.41	1.66	0.04
SE18, 500	49	1.51	3.07	0.02	n.d.	0	n.d.	n.d.	0	n.d.
NE8, 200	168	11.78	7.02	0.12	4.22	2.52	0.04	3.67	2.19	0.03
NE26, 500	97	5.50	5.68	0.05	1.82	1.88	0.02	1.23	1.28	0.01
NE45, 1000	126	7.55	5.98	0.07	3.06	2.42	0.03	2.35	1.86	0.02
NW30, 1000	204	10.03	4.92	0.11	4.61	2.26	0.04	4.13	2.03	0.04
NW18, 500	918	26.90	2.93	0.26	24.39	2.66	0.20	21.95	2.39	0.19
SE10, 300	2137	12.03	0.56	0.14	1.14	0.05	0.01	1.07	0.05	0.01
SE19, 500	1287	63.15	4.91	0.62	30.42	2.36	0.26	26.65	2.07	0.24
SW6, 300	1056	91.13	8.63	0.93	25.43	2.41	0.22	21.57	2.04	0.20
NE27, 500	251	18.66	7.43	0.17	12.85	5.12	0.10	11.06	4.41	0.09
NW6, 200	377	14.37	3.81	0.17	3.06	0.81	0.03	1.72	0.46	0.02
SE30, 1000	161	5.43	3.37	0.06	2.98	1.85	0.03	2.69	1.67	0.03
SW10, 500	296	14.71	4.96	0.15	6.63	2.24	0.06	6.04	2.04	0.05

Total and extractable amounts of Pb shown in mg/kg, % extraction efficiency and mg/L.

Highlighted in yellow are samples over the guideline values – Pb 450 mg/kg.

Table 3.8 Soil/simulated gut (BGS method) extracts; Zn.

Sample name	Zn	Stomach			Int1			Int2		
	Total	mg/kg	%	mg/L	mg/kg	%	mg/L	mg/kg	%	mg/L
SW12, 600	198	47.71	24.07	0.49	12.36	6.24	0.11	6.07	3.06	0.06
SW14, 700	235	61.74	26.26	0.61	14.59	6.20	0.13	10.94	4.65	0.10
SW16, 1000	293	45.51	15.55	0.45	9.99	3.42	0.09	4.86	1.66	0.05
SE18, 500	107	13.81	12.85	0.15	n.d.	0	n.d.	n.d.	0	n.d.
NE8, 200	232	80.88	34.85	0.80	18.96	8.17	0.16	13.55	5.84	0.12
NE26, 500	130	20.42	15.70	0.20	1.58	1.21	0.01	n.d.	0	n.d.
NE45, 1000	192	44.45	23.12	0.44	8.22	4.27	0.07	3.72	1.93	0.03
NW30, 1000	267	61.51	23.00	0.65	14.66	5.48	0.14	11.46	4.28	0.11
NW18, 500	1213	472.75	38.98	4.65	211.57	17.45	1.74	178.99	14.76	1.53
SE10, 300	1419	193.97	13.67	2.18	48.39	3.41	0.47	37.74	2.66	0.38
SE19, 500	817	244.22	29.89	2.40	81.66	10.00	0.69	61.85	7.57	0.55
SW6, 300	1511	757.36	50.11	7.87	348.07	23.03	3.15	284.80	18.84	2.70
NE27, 500	342	104.28	30.45	0.97	34.61	10.11	0.27	25.37	7.41	0.21
NW6, 200	470	85.91	18.28	0.99	28.42	6.05	0.29	17.79	3.79	0.19
SE30, 1000	306	96.17	31.47	1.06	26.41	8.64	0.25	19.59	6.41	0.19
SW10, 500	463	151.12	32.67	1.50	42.40	9.17	0.36	31.97	6.91	0.28

Total and extractable amounts of Zn shown in mg/kg, % extraction efficiency and mg/L.
Highlighted in yellow are samples over the guideline values –Zn 300 mg/kg.

3.3.3 – Soil/DMEM extract

Table 3.9 shows levels of Cu, Ni, Pb and Zn extracted from the 16 selected Byker samples using DMEM (human cell culture medium). Amounts of extractable Cu ranged from 0~12% with sample SW6,300 again exhibiting levels in mg Cu/kg over the 130 mg/kg guideline value (139.59 mg/kg). SW6,300 also showed the highest level of Cu (in mg/kg) available in all 3 other methods used. Levels of extractable Ni were low, ranging 0~2.5% (highest value 1.13 mg Ni/kg, sample NW6,200). NW6, 200 also demonstrated the highest extractable value with the RIVM extract, one of the higher values with the water extract but was not one of the higher values using the BGS extract.

Levels of extractable Pb were low, being 0~2.5% of the total levels (highest value 2.3 mg Pb/kg, sample SW16,1000). The highest and lowest values using the DMEM extraction method were not similar to any of the other 3 extract methods used.

Levels of extractable Zn were 0~1.3% (highest value 15.06 mg Zn/kg, sample NW18,500). SW6,300 also showed a high extractable value, similar to the BGS extract method results.

The generally low level of metal availability was most likely due to the physiological pH of the DMEM.

Table 3.9 Soil/DMEM extracts.

Sample name	Cu in mg/kg		%	Cu	Ni in mg/kg		%	Ni	Pb in mg/kg		%	Pb	Zn in mg/kg		%	Zn
	Total	DMEM		mg/L	Total	DMEM		mg/L	Total	DMEM		mg/L	Total	DMEM		mg/L
SW12, 600	53	2.88	5.47	0.29	28	0.65	2.34	0.07	252	0.78	0.31	0.08	198	n.d.	0	n.d.
SW14, 700	74	6.45	8.77	0.65	24	0.41	1.76	0.04	269	1.09	0.41	0.11	235	n.d.	0	n.d.
SW16, 1000	88	2.34	2.65	0.23	36	0.69	1.91	0.07	266	2.30	0.86	0.23	293	n.d.	0	n.d.
SE18, 500	40	2.16	5.37	0.22	29	0.62	2.14	0.06	49	1.24	2.53	0.12	107	n.d.	0	n.d.
NE8, 200	80	6.89	8.62	0.69	22	0.43	1.96	0.04	168	0.70	0.42	0.07	232	n.d.	0	n.d.
NE26, 500	37	2.48	6.64	0.25	13	0.11	0.86	0.01	97	1.04	1.08	0.10	130	n.d.	0	n.d.
NE45, 1000	80	6.39	8.01	0.64	22	0.54	2.47	0.05	126	0.64	0.51	0.06	192	n.d.	0	n.d.
NW30, 1000	80	5.88	7.32	0.59	20	0.45	2.24	0.05	204	n.d.	0	n.d.	267	n.d.	0	n.d.
NW18, 500	285	9.38	3.30	0.94	90	1.07	1.20	0.11	918	0.42	0.05	0.04	1213	15.06	1.24	1.51
SE10, 300	3377	9.44	0.28	0.94	94	0.54	0.58	0.05	2137	0.58	0.03	0.06	1419	0.95	0.07	0.10
SE19, 500	1129	36.65	3.25	3.67	51	0.40	0.78	0.04	1287	0.40	0.03	0.04	817	0.62	0.08	0.06
SW6, 300	1205	139.59	11.59	13.96	72	0.80	1.11	0.08	1056	0.26	0.03	0.03	1511	6.55	0.43	0.65
NE27, 500	135	8.42	6.23	0.84	26	0.55	2.13	0.06	251	0.44	0.17	0.04	342	0.28	0.08	0.03
NW6, 200	532	47.26	8.89	4.73	82	1.13	1.37	0.11	377	0.56	0.15	0.06	470	0.10	0.02	0.01
SE30, 1000	1046	10.00	0.96	1.00	23	0.44	1.92	0.04	161	0.46	0.28	0.05	306	n.d.	0	n.d.
SW10, 500	144	8.37	5.82	0.84	26	0.28	1.06	0.03	296	1.02	0.34	0.10	463	1.21	0.26	0.12

Total and extractable amounts of Cu, Ni, Pb and Zn shown in mg/kg, % extraction efficiency and mg/L.
Highlighted in yellow are samples over the guideline values – Cu, 130 mg/kg; Ni 50 mg/kg; Pb 450 mg/kg; Zn 300 mg/kg. n=3

3.3.4 Selected soil physical and chemical properties

Some soil properties (particle size, pH, TOC and CEC) of the 16 selected samples are outlined in Table 3.10.

Table 3.10 Basic physical soil data of the 16 sub-set of samples

Sample name	Particle Size in %				pH	TOC%	CEC
	Coarse sand	Fine sand	Silt	Clay			
SW12, 600	18.48	18.72	46.30	16.50	6.25	6.27	17.46
SW14, 700	35.75	30.82	23.93	9.49	6.8	6.96	17.05
SW16, 1000	21.52	21.79	37.41	19.28	6.77	6.23	21.73
SE18, 500	23.17	27.17	41.67	7.98	6.59	8.41	18.50
NE8, 200	33.23	24.98	29.63	12.17	6.92	4.56	14.73
NE26, 500	28.04	37.18	23.96	10.82	6.51	2.29	10.22
NE45, 1000	30.22	26.80	29.32	13.66	6.55	6.71	19.14
NW30, 1000	27.85	31.57	30.57	10.01	6.6	6.15	16.03
NW18, 500	46.14	24.93	22.65	6.28	6.93	11.61	22.70
SE10, 300	20.22	18.32	24.16	37.30	7.27	3.14	27.49
SE19, 500	35.89	25.71	23.14	15.25	6.83	4.40	16.33
SW6, 300	51.61	24.73	16.79	6.87	7.01	6.48	14.68
NE27, 500	24.52	26.37	35.73	13.38	5.69	7.37	17.15
NW6, 200	49.97	24.75	15.50	9.76	6.47	4.32	11.14
SE30, 1000	37.54	20.50	28.20	13.76	6.72	5.85	17.78
SW10, 500	46.01	19.08	26.50	8.41	6.9	7.04	16.51
MEAN	33.13	25.21	28.45	13.18	6.67	6.11	17.41
MEDIAN	31.72	24.96	27.35	11.49	6.75	6.25	17.10

particle size, pH, TOC and CEC (to 2dp)

Highest levels of clay were seen in samples SE10,300 and SW16,1000 whereas the highest levels of sand in were seen in SW6,300, NW6,200 and NW18,500. The highest level of TOC was seen in SE18,500 and the highest level of CEC was seen in SE10,300.

3.3.5 Statistical analysis of factors influencing metal extractability

Using regression analysis, no significant **($P<0.05$)** correlation was seen between extractability of copper, using any of the 4 extracts techniques, and "total" metal and/or any of the soil properties (pH, TOC, CEC and particle size). Significant correlation ($P<0.05$) was found between the "total" levels of Ni and both the extractable levels of Ni in the Int2 fraction (of the BGS extract method) and the DMEM extract method. Both gastrointestinal simulation methods (RIVM and BGS stom, int1 and int2) showed significant ($P<0.05$) correlations with "total" lead levels and the particle sizes (sand, silt and clay) of the samples. Levels of Zn extracted from samples by both gastrointestinal simulation methods (RIVM and BGS stom, int1 and int2) were significantly ($P<0.05$) correlated to total levels of Zn and soil particle size (sand, silt and clay) of the samples. Levels of Zn found to be extractable using DMEM were significantly ($P<0.05$) related to "total" levels of Zn and TOC in the samples.

3.3.6 Graphical comparison of total and extractable levels of Cu, Ni, Pb and Zn.

These graphs are included for visual comparison purposes and were selected to emphasise the levels of metal extractability compared to total levels of metal.

Figures 3.2 and 3.3 present levels of total and extractable (bioaccessible) copper for four control samples and four heavily contaminated samples compared to the guideline value (130mg/kg for Cu). NB Graph scales are NOT all identical. Figures 3.4 and 3.5 show total and extractable levels of nickel and lead (respectively) for four contaminated soils (guideline values 50 mg/kg and 450 mg/kg respectively) and figures 3.6 and 3.7 present total and extractable levels of zinc (guideline value 300 mg/kg) from eight contaminated soils. "Total" and "BGS" samples give a single value (analysis done at commercial laboratories with stringent internal standards applied) whereas water extractable, "RIVM" and "DMEM" values shown are means (n=3) +/- SEM. Graphs of all other samples are available in Appendix 3.

COPPER

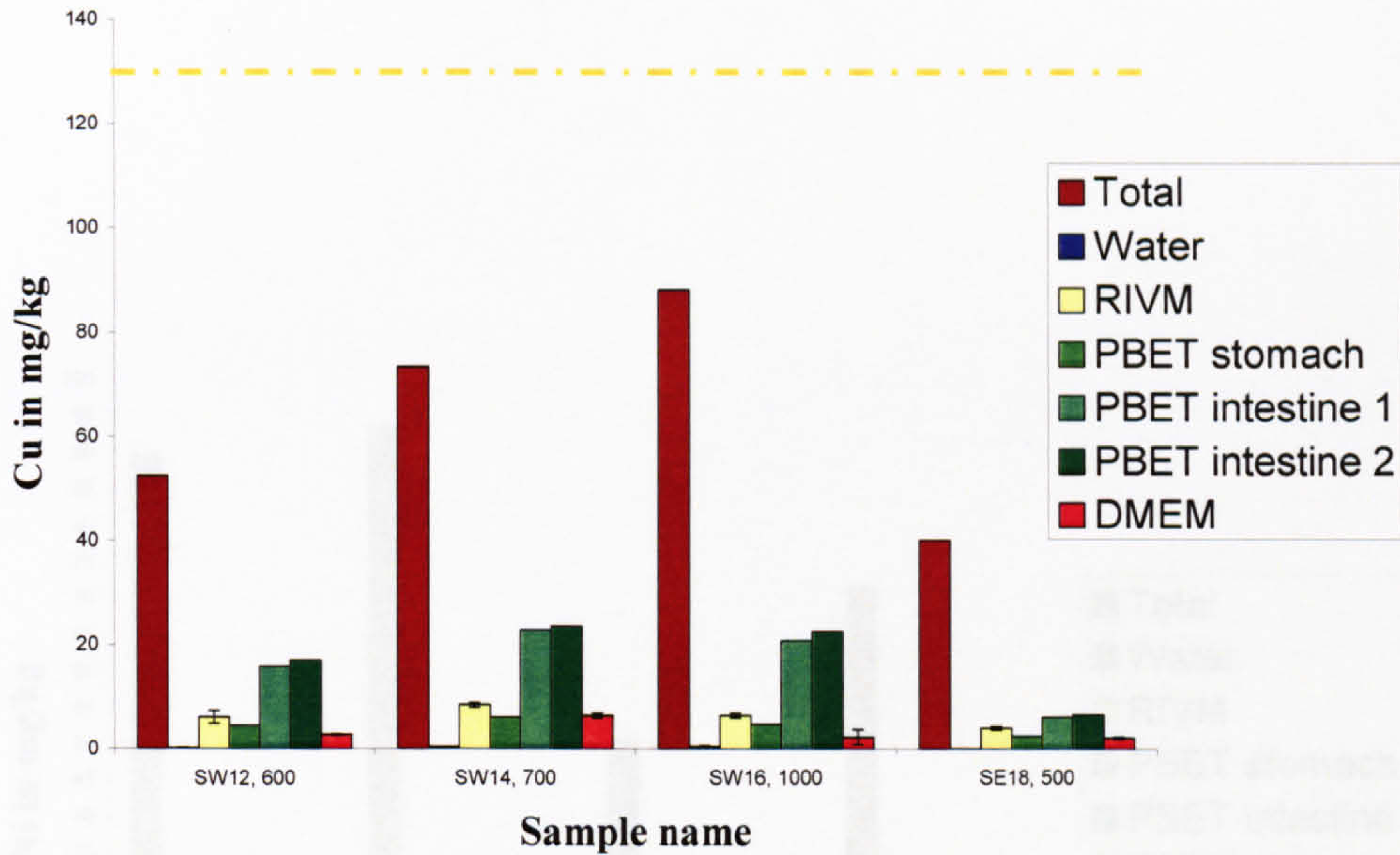


Figure 3.2 – Levels of Cu in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed yellow line indicates guideline value for Cu – 130 mg/kg.

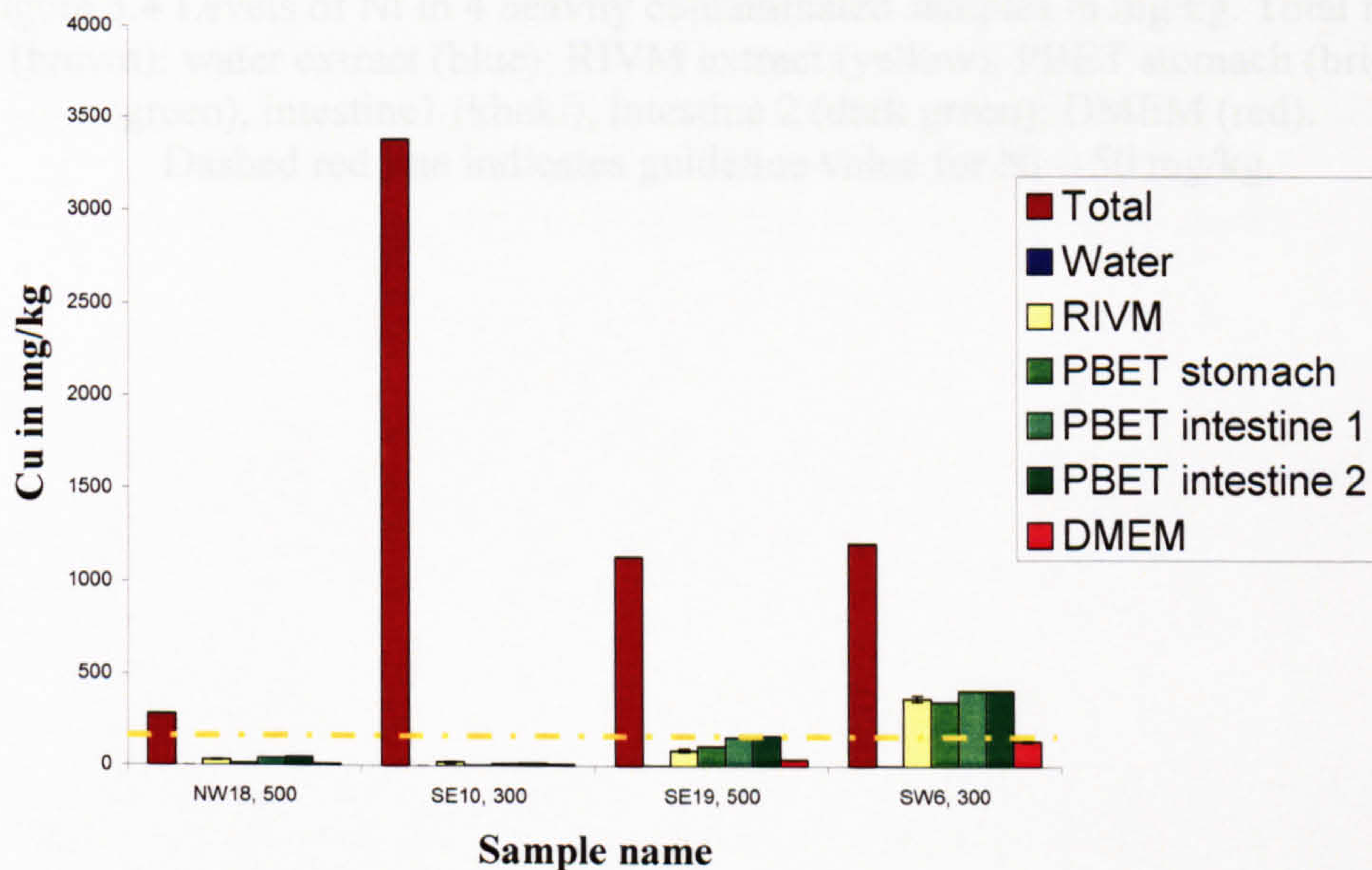


Figure 3.3 - Levels of Cu in 4 heavily contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed yellow line indicates guideline value for Cu – 130 mg/kg.

NICKEL

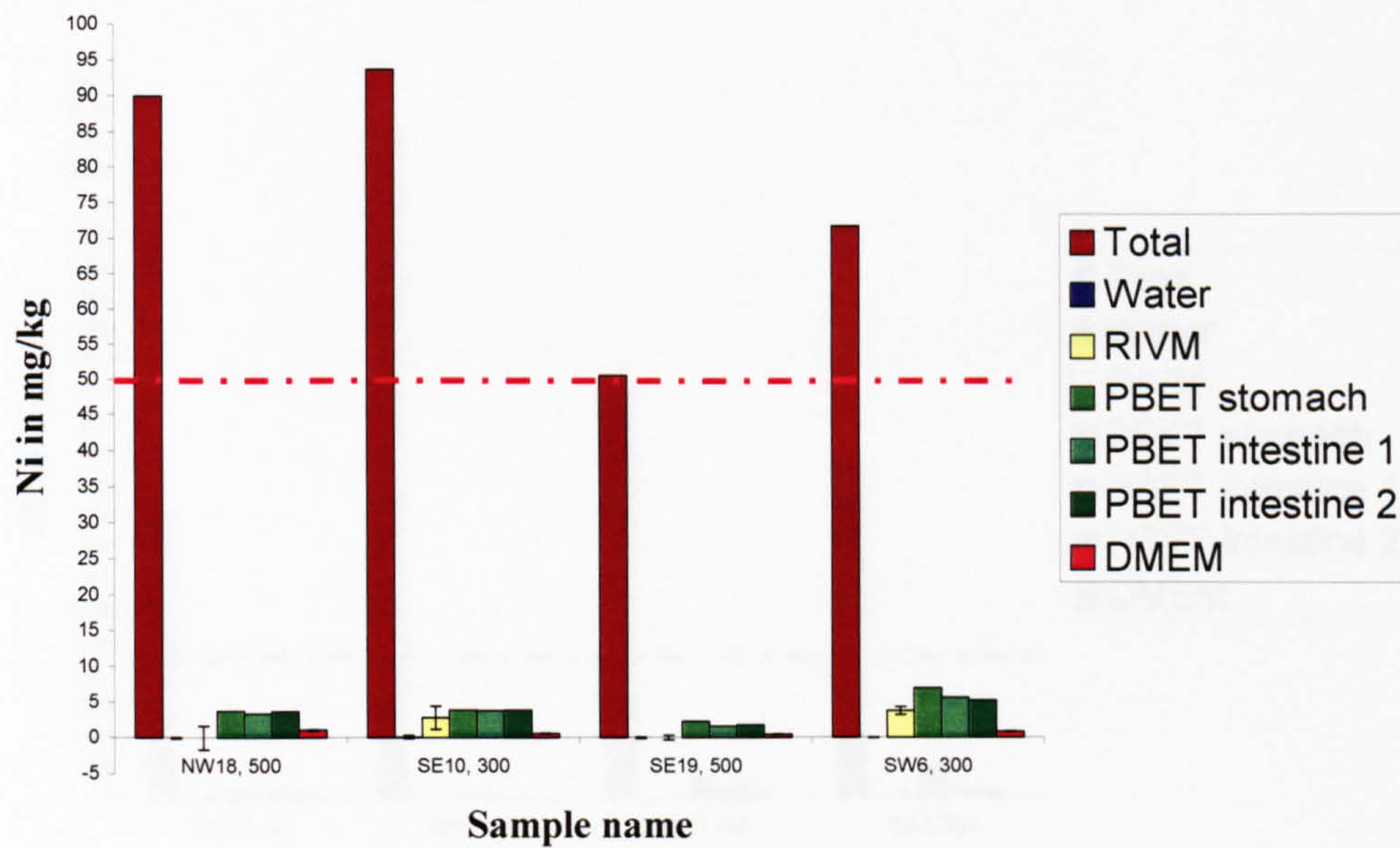


Figure 3.4 Levels of Ni in 4 heavily contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed red line indicates guideline value for Ni – 50 mg/kg.

LEAD

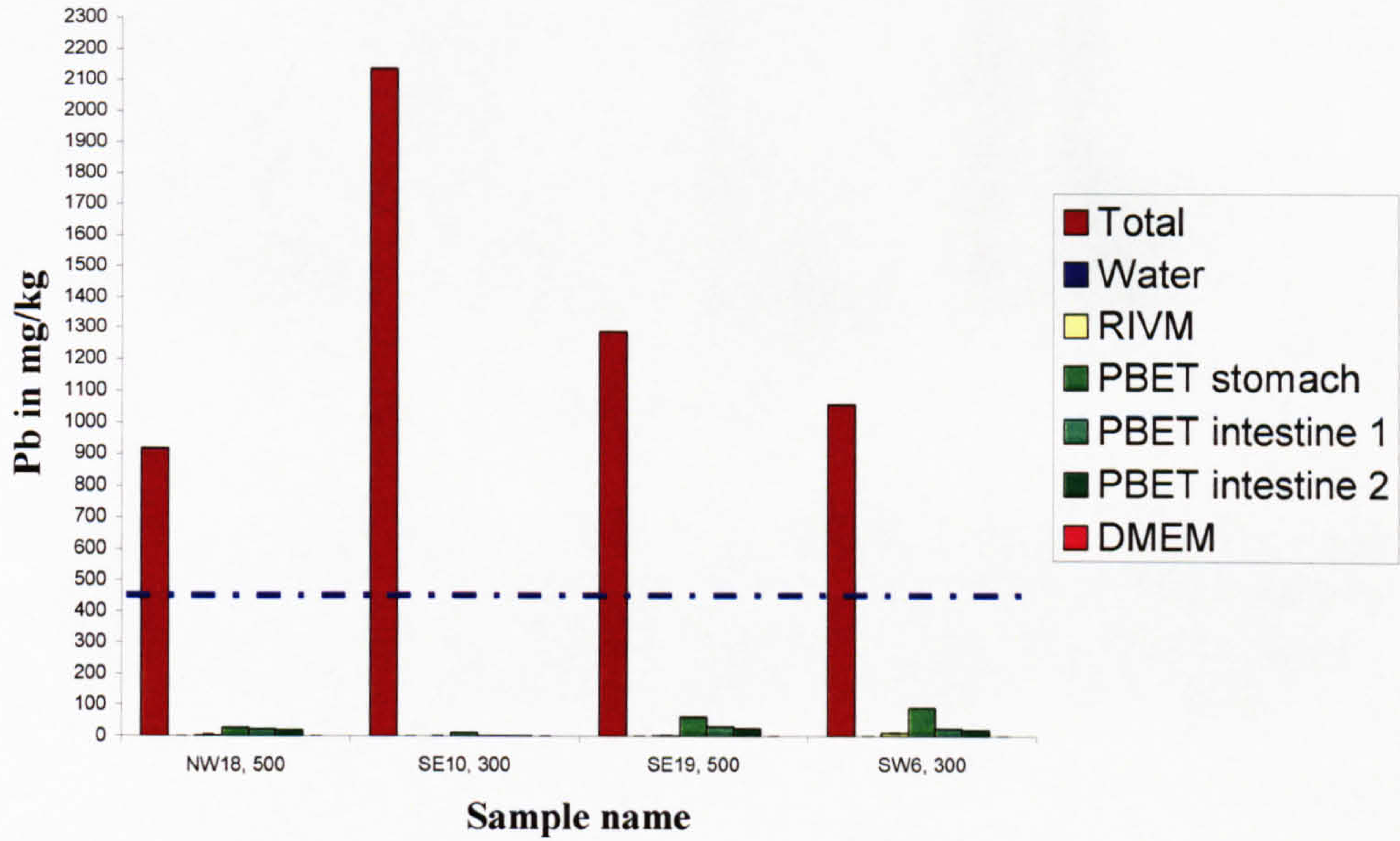


Figure 3.5 Levels of Pb in 4 heavily contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed blue line indicates guideline value for Pb – 450 mg/kg.

ZINC

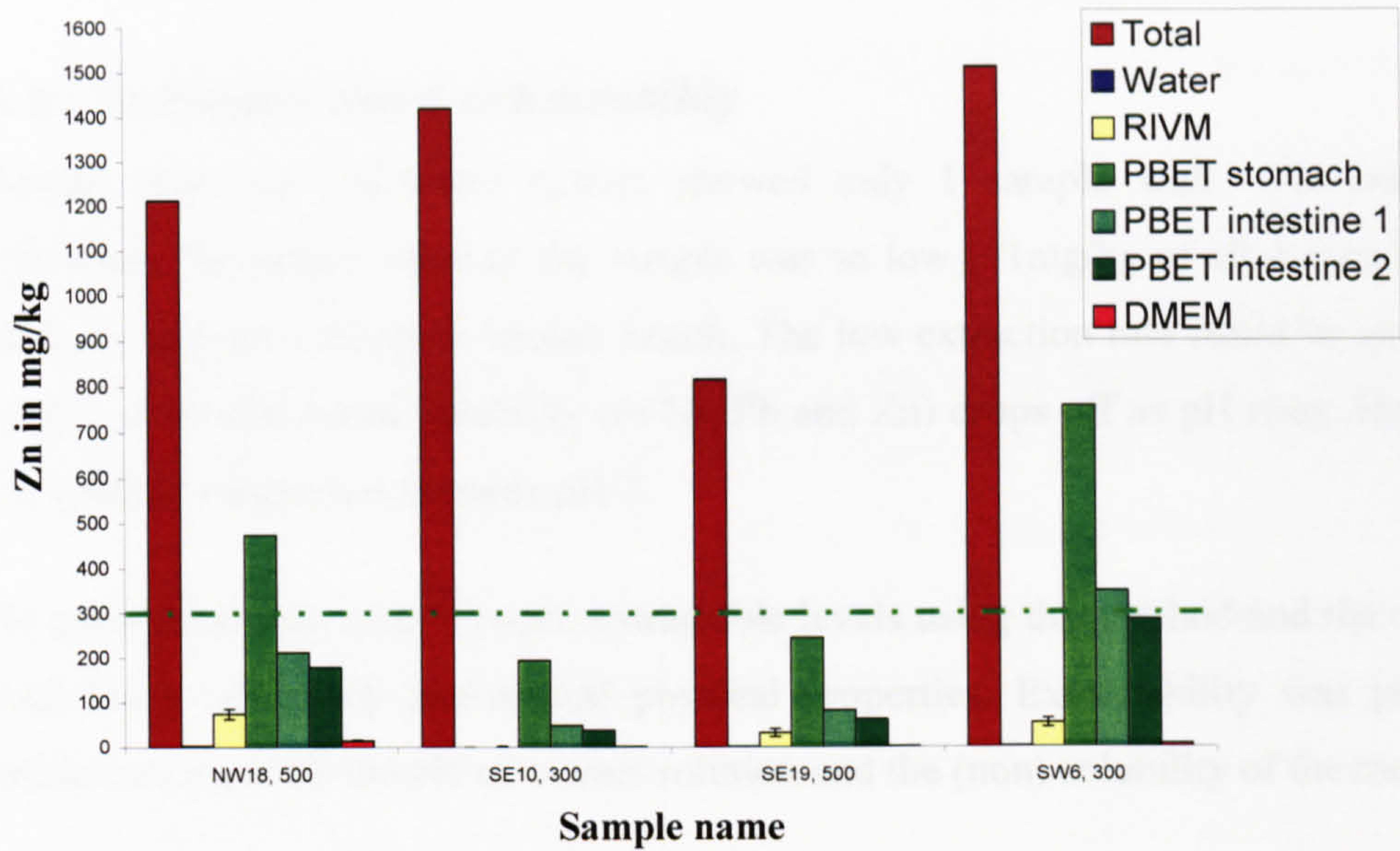


Figure 3.6 - Levels of Zn in 4 heavily contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed green line indicates guideline value for Zn – 300 mg/kg.

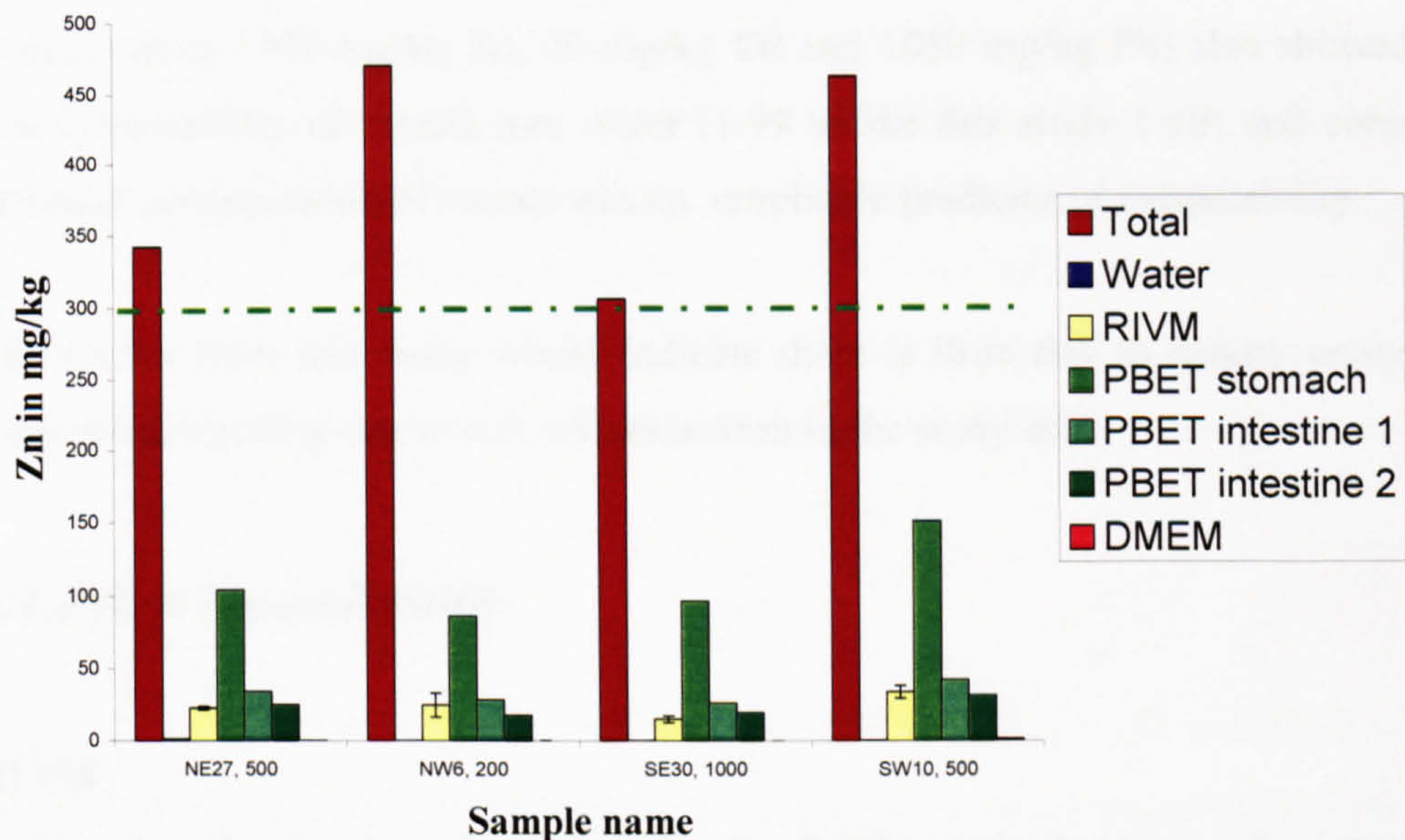


Figure 3.7 Levels of Zn in 4 contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed green line indicates guideline value for Zn – 300 mg/kg.

3.4 DISCUSSION

3.4.1 Soil/water metal extractability

Results from the soil/water extract showed only 1 sample with >1% extraction efficiency, the actual value of the sample was so low (<1mg/kg of all 4 metals), it is unlikely to pose a threat to human health. The low extraction rate could be attributed to pH, given that metal solubility (of Ni, Pb and Zn) drops off as pH rises. However, Cu solubility increases towards pH 7.

No correlation was seen between extractable levels using this method and the original total levels of metals and/or soil physical properties. Extractability was probably influenced more by the pH of extract solution and the (non) solubility of the metals.

It was important to assess the potential leaching into water as the study area contains several waterways – including the rivers Tyne and Ouseburn, with ecosystems that may be affected by heavy metals. The Ouseburn valley contained (until remediation in 2004-5) a source of white lead, a form of the metal known to be highly insoluble. Aruoja *et al.*, (2004) found that soils sampled from near lead and zinc smelters in France (up to 1390 mg/kg Zn, 20 mg/kg Cd and 1050 mg/kg Pb) also showed very low extractability of metals into water (1:99 unlike this study 1:10) and concluded that total concentration of metals was an unreliable predictor of extractability.

The results from this study would indicate there is little risk to nearby ecosystems from metal leaching due to soil contamination in the study area.

3.4.2 Oral bioavailability

RIVM

The results of extraction of metals using the RIVM method were much more varied than those obtained using the soil/water extraction method. This was probably due to changes in pH in the solutions used. The availability of Cu ranged from <1% to ~30%. The most notable point is the contrast between the 2 samples at either end of the extraction efficiency range. SW6,300 and SE10,300 both had very high total levels of

Cu; in the former sample ~30% was available (SW6, 300) whereas in the latter sample virtually none (<1%) was (SE10,300). This result could potentially be explained by the high clay content of sample SE10,300 (~37%) and the high sand/low clay of sample SW6,300 (Rowell, 1994). Analysis of levels of Ni showed all samples exhibited consistently low extractability (ranging from 0-~6.5%). Levels of extracted Pb were even lower, with all samples showing <1% extractability. Zn extractability ranged from 0 to ~7.5%; whilst the range was more varied than Pb and Ni, the actual values were still low (max ~75 mg Zn/kg). Again, the influence of pH on metal solubility may provide an explanation as to the low solubility although other studies have found much greater ranges of lead availability. For example Sips *et al.*, (2001) found that Pb bioaccessibility ranged from 6-72% in samples tested (speciation and soil type were thought to be contributory factors, pH was not) whereas Oomen *et al.*, (2002) found a 11-66% extractable range (Pb).

Limitations and drawbacks of using the RIVM method include that it has not been validated for any compounds or chemicals except Benzo(a)pyrene As, Cd and Pb and even then the validation process (which consisted of comparison to other bioaccessibility tests) was not in comparison to animal studies. Although the method mimics different digestive compartments, only one sample is taken for analysis, at the end of the protocol when the pH has been raised to a physiological level. It is also technically more difficult, more time consuming and more expensive (a large range of chemicals is required) than other methods used internationally (e.g. the BGS PBET). Only one sample (SW6,300 for Cu) showed an extractable level (in mg/kg) over the guideline values, using the RIVM method and would therefore require further risk assessment or remediation.

BGS PBET

The Cu Stom fraction showed 0-30% extractability and as with the RIVM extract method results, sample SW6,300 showed the highest % extraction and actual value (in mg Cu/kg), whilst sample SE10,300 showed the lowest % extraction. The Int1 fraction had 0-35% recovery with sample SW6,300 still showing the highest % extraction. The Int2 fraction values were very close to Int1 but all slightly higher. The increase in pH appears to have increased the amounts (mg Cu/kg) extracted from all samples. The opposite effect was seen with Ni, Pb and Zn; as the pH increased the

extractability dropped (Stom>Int1>Int2). Although with Zn, 2 samples still showed extractability values over the guideline levels; SW6,300 (Stom and Int1) and NW18,500 (Stom). These results would indicate that when assessing the extractability of Cu it is important to use a method with a physiological pH.

Oomen *et al.* (2002) used the SBET technique (Stom fraction of the PBET) and found 56-91% Pb was extractable from the soils used; that study did not further investigate the influence of metal speciation or soil properties on extractability as its main focus was on comparing the results from five gastrointestinal analogue methods.

Soils from the Nuns Moor allotments (Newcastle-upon-Tyne) were analysed for Pb (and As) and using the BGS PBET, a range of 7.52 to 8.38% Pb was found to be extractable (Newcastle City Council, 2004). On the basis of this evidence (and full desk study), rather than remediate the site, health advice and improved hygiene facilities were provided at substantially lower cost (personal communication, Phil Hartley, Newcastle City Council).

In total, three samples were found to have extractable levels of metal (in mg/kg) above the guideline values; SE19,500 (Int1 and Int2 fractions) were above guideline for Cu and NW18,500 (Stom) was above the guideline value for Zn. SW6,300 Stom, Int1 and Int2 fractions were all above the Cu guideline whereas only Stom and Int1 were above the Zn guideline. The RIVM method also found SW6,300 to exceed the guideline for Cu.

Limitations of the BGS method are like those for the RIVM. Only a limited range of chemicals and compounds have been validated using this method including As and Pb but unlike the RIVM method, animal testing has been carried out.

Other simulated gut studies

No previous work appears to have been performed on the bioaccessibility of bioavailability of Cu or Zn using gastrointestinal analogues, making the results in this report entirely novel. two separate studies have investigated Ni bioavailability; Hamel *et al.*, (1998) examined differences in levels of extractable Ni (and Pb, As, Cd and Cr) due to solid:liquid ratios (100:1 up to 5000:1were investigated) in synthetic gastric

fluids, levels found were ~11-14% in one soil and ~23-40% in another. Ollson, (2003) investigated Ni availability *in vitro* and *in vivo*. No actual numerical data was provided for the *in vitro* experiment but the results were found to be almost comparable to the *in vivo* experiment (2.1-3.9% available range found)

The majority of work examining the gastrointestinal bioavailability of metals has focused on As (Environment Agency, 2002a; Hamel *et al.*, 1999; Oomen *et al.*, 2002; Rodriguez & Basta, 1999), Pb (Hamel *et al.*, 1999; Oomen *et al.*, 2002; Ruby *et al.*, 1993), Cd (Basta *et al.*, 2005; Oomen *et al.*, 2002; Schroder *et al.*, 2003) and Cr (Hamel *et al.*, 1999; Skowronski *et al.*, 2001). No study found gastrointestinal bioavailability of As using *in vitro* methods to be approaching 100%.

A record of only one actual human feeding study could be found. (Maddaloni *et al.*, 1998) fed six adult volunteers with lead contaminated soil (in a capsule); those who consumed the soil whilst fasting were found to absorb 26.2% +/-8.1 into the blood stream, whereas those who consumed the soil with food, only 2.52%+/- 1.7 was found to have been absorbed into the blood.

3.4.3 DMEM extract

The percentage extractability of all four metals, using the DMEM extraction method, followed a pattern similar to the gastrointestinal analogues. Cu ranged from 0-12% extractability with SW6,300 showing the highest % extracted (actually above guideline value) and SE10,300 the lowest. Both Ni and Pb showed extremely low levels of extractability (0-2.5%), probably due to the physiological pH of the DMEM fluid. Zn availability ranged from 0-15% with SW6,300 and NW18,500 showing (like BGS Stom) the (relatively) highest levels.

As with gastrointestinal analogues, both Cu and Zn showed much greater ranges of extraction efficiency. This may be due to the influence of digestive enzymes in the gastrointestinal extracts and some unknown factors in DMEM growth medium that complex or select for, Cu and Zn as both are micronutrients, necessary for human survival at low levels.

Only one previous study has used cell culture media to examine the extractability of metals from soils. Langley-Turnbaugh *et al.* (2005) used DMEM/F12+CCS growth media to determine the "biosolubility" of Al, As, Cu, Mn, Ni, Pb and V from soils (but not Zn). Only Cu was found to be biologically available and Pb and Al did not appear to be soluble in the biological medium. The findings of Langley-Turnbaugh *et al.* (2005) would appear to be similar to the results found here, with Cu being more biologically extractable than Ni and Pb.

The main flaw with this method is that at no stage has any (peer reviewed) validation of results against animal tests or any simulated *in vitro* methods been performed. The relevance of these results cannot be totally dismissed just because the method is novel; the results appear to follow the same pattern as the gastrointestinal analogues and given that the protocol is quicker, cheaper and less complex to perform, it may have merit as a screening method.

3.4.4 Soil physical properties

The mean (6.67) and median (6.75) pH of the Byker subset of samples were slightly higher than those reported in Mellor (2001) for Tyneside soils (mean 6.1 and median 6.0) whereas the TOC was substantially lower (Mellor – mean 18%, median 17%, this study mean 6.11%, median 6.25%). The extractability of metals from soils is commonly acknowledged to be dependant on desorption into soil solution from the surfaces of colloidal material e.g. organic matter, clay fraction and Fe and Mn oxides (Dijkstra *et al.*, 2004; Moreno *et al.*, 2005). Based on the physical soil data of the 16 samples, it could be assumed that the lowest bioavailability of the 4 metals would be exhibited by SE10,300, SW16,1000 (37.3% and 19.28% clay respectively) and NW18,500 (11.61% TOC). Whilst SE10,300 stands out as having very low % extractable levels of metal (e.g. lowest Cu in DMEM extract), neither of the other 2 samples appeared different. Conversely, samples with the lowest % of clay and highest levels of sand would be expected to exhibit the greatest levels of extractable metal. NW18,500 and SW6,300 have the lowest levels of clay (and very high levels of sand) and both exhibit high levels of extractability of zinc; SW6,300 also has the highest percentage of extractable copper.

3.4.5 Statistical analysis and graphical comparison

The statistics bear out the hypothesis that the actual pH of the soils is not significantly related to physiological extractability of metals whereas the pH of the extract solution has more influence. Sips *et al.*, (2001) also found no connection between soil pH and bioaccessibility of Pb using the RIVM extract method. No correlations were seen between the available data for copper and the soils and its extractability using any method. Significant correlations were seen between Int2 (BGS PBET) and DMEM levels of extractable Ni and total Ni. This result may show that at higher pH's, extractable and total Ni can be related but the actual levels and extraction percentages are still very low (<2.5% and <8.6% respectively). Examination of the Pb data showed that for both gastrointestinal analogues, extractable levels could be related to total lead and the soil particle size measurements. Zn data also showed a relationship between extractable levels using the gastrointestinal analogues and total Zn and soil particle size. The only extract method that showed a correlation to TOC (and total Zn) was DMEM.

The importance of site-specific investigations into the bioavailability of metal contamination is best illustrated by Figures 3.3 (Cu) and 3.6 (Zn). More specifically, the contrast between the availability of Cu and Zn from SE10,300 and SW6,300, both have high total levels of metal; SW6,300 shows extractable levels above guideline values (i.e. high bioavailability) whereas SE10,300 shows minimal extractability of its metal contamination, regardless of the very high "total" levels present in the sample. The graphs also illustrate how low the percentage extractions of these metals are using physiological based extraction techniques compared to "total" amounts.

3.4.6 Summary

Using the results generated from this work, it could be safely assumed that the soils tested are unlikely to contaminate local waterways (e.g. the River Tyne and its tributary the Ouseburn River) with Cu, Ni, Pb and Zn and the ecosystems contained in them. Both of the gastrointestinal analogues performed and the leaching into physiological fluid found that of the 16 samples, only three (SW6,300, NW18,500 and SE19,500) may pose a threat to human health, with levels of Cu and Zn above

guideline values (that is, if the guideline values used in this study, taken from the withdrawn ICRCL regime, were applied, no CLEA values currently exists for Cu and Zn) with prolonged exposure.

The advantages of using *in vitro* methods as a screening tool to investigate extractable levels of metals from contaminated soils in order to assess risk include significantly lower costs, timescales, greater reproducibility and fewer ethical dilemmas. Current limitations are that methods need to be calibrated against *in vivo* (human or animal) tests and even then it is not possible for these methods to safely be assumed to be correct for mixtures of contaminants and all soils. In addition, it is not known whether these extract solutions would be suitable for use in bioassays (tests using biological organisms as surrogates for humans or ecosystems, e.g. bacteria or invertebrates) in order to be able to further assess risk by investigating actual toxicity.

Gastrointestinal analogues are likely to increase in popularity as risk assessment tools for contaminated land and several methods are currently being commercially marketed in the UK, including the BGS PBET.

CHAPTER 4 – Assessing the potential of a novel *in vitro* human cell culture test to determine the toxicity of contaminated soil

4.1 INTRODUCTION

Given that the main thrust of contaminated land legislation in the UK and internationally is the protection of human health, it follows that methods to indicate the actual biological long term toxic effect of contaminated soils to humans are highly desirable and necessary. Several major limitations of the CLEA model (and contaminated land guidance internationally) include –

- Soil guideline values are based on animal studies and human biomonitoring studies (mostly retrospective studies)
- Guideline values do not exist, or are long outdated, for a wide range of contaminants (for example, Cu and Zn are not currently addressed by the CLEA model).
- Guidance states that levels of contamination above SGV's require further assessment but does not specifically define that what form that assessment should take.

These gaps in current knowledge and practices would make the development of model systems, preferably human, to directly look at the effect of contaminated soil to man in order to assess toxicity, highly desirable. Given that it is ethically unsound to test directly on humans, *in vitro* use of human cell lines suggests itself as a practical way forward. Factors to be taken into consideration when designing/selecting such a test include the routes of exposure, bioavailability and how to judge the actual risk. From a more practical stance, any test must ideally be robust and easily reproducible as well as cheap, quick and ethical.

4.1.1 Current regimes used to assess the toxicity of substances to humans

No single toxicity assay is capable of detecting the full range of toxic responses the human body produces. Various physiological targets and many different complex toxicokinetic mechanisms are usually involved so it is important to test for a range of factors including cytotoxicity (acute poisoning leading to cell death) and more subtle toxic responses such as DNA damage i.e. genotoxicity.

The most common user of human toxicity assays is the pharmaceutical industry; obligated to use a battery of tests on any/all products to assess cytotoxic and genotoxic potential. Official guidance outlining methods to be used for genotoxicity testing are described in the International Conference of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use documents (ICH, 1995; ICH, 1997). This guidance is internationally adhered to and dates from the late 1990's. No equivalent guidance appears to exist for cytotoxicity testing.

Cytotoxicity testing

There exists a huge variety of cell parameters that can be measured to assess cell viability; for example measurement of cell membrane integrity, cell metabolic competence and detoxification capacity. Cytotoxicity testing is also necessary when performing genotoxicity work to ensure positive results in a genotoxicity assay are not falsely ascribed (i.e. to ensure DNA damage seen are not false positives related to apoptosis or necrosis). A wide range of cytotoxicity/cell viability tests are available, looking at different aspects of cell function that are required for viability. Older generations of tests used expensive chemicals and radioactivity and tended to measure physical parameters such as cell membrane integrity (e.g. trypan blue dye exclusion live/dead); where as the current generation looks at metabolites (e.g. ATP, NADH, glucose uptake) and other biochemical indices of cytotoxicity including total protein and cell proliferation. With cytotoxicity testing, in addition to wanting a robust, reproducible and accurate assay, it is important to be able to automate in order to have a high throughput system and easy to measure endpoints using cheap non-toxic chemicals.

Genotoxicity testing

The genotoxicity test battery recommended in the pharmaceutical guidelines includes both bacterial screening and mammalian *in vitro* and *in vivo* protocols (to ensure the effects of metabolic activation of genotoxins are taken into account). More specifically the main *in vitro* tests should include:-

1. A bacterial reverse mutation assay (the gold standard being the Ames test)
2. Gross chromosomal damage (i.e. sister chromatid exchange)
3. Chromosomal number change
4. Chromosomal abnormality (analysis of micronuclei - MN)

A set of *in vivo* tests should also be performed which include animal tests looking for chromosomal damage, aberrations and analysis of micronuclei (usually using blood or bone marrow).

Other commonly used and validated genotoxicity assays include fluorescent in situ hybridisation (FISH) and the Comet assay. The former method involves looking at DNA strand breakage; although it is more sensitive than examining crude chromosomal change it is highly expensive and time consuming. The latter, the Comet assay, is a technique that is used to quantify DNA strand breaks – a major end point of short-term genotoxicity assays.

4.1.2 The Comet assay

The single cell gel electrophoresis assay (SCGE), more commonly known as the "Comet" assay (due to the appearance of cells) is a sensitive and subtle assay that can be used to quantify and assess DNA damage in single cell preparations of any eukaryotic cells. As outlined in section 1.7, the technique was first described in 1984 (Ostling & Johanson, 1984) and was adapted to the current alkaline form in 1988 (Singh *et al.*, 1988). It is used in a wide range of applications including genotoxicity testing (of known and new compounds), human and ecological biomonitoring and research into DNA damage and repair mechanisms.

The method consists of pre-treating a cell population, fixing the cells in agarose on a microscope slide and then lysing the cells with a combination of salts and detergents so a nucleoid structure remains, subjecting the preparations to electrophoresis under

alkaline conditions, staining the slides with a fluorescent DNA-binding dye and subsequently visualising the DNA. The assay is sensitive in a dose dependant manner. The actual mechanism leading to formation of a "comet" tail is by several routes; oxidation of guanine (Gedik *et al.*, 1998), interruption of DNA supercoils, interruption of excision repair to DNA adducts and depurination of N-7 adducts during alkali unwinding leading to ssDNA (McKelvey Martin *et al.*, 1993). Results cannot be treated like normal electrophoresis where migration is proportional to fragment size; tail length is only an accurate measure of dose response, up to a point. Apoptotic cells can also be subjected to this treatment; therefore it is very important to run cytotoxicity tests in parallel. . The assay has a limited working range, as when DNA is to highly damaged, no measurement is possible. The assay and all its potential uses and methodological detail have been extensively reviewed (see Table 4.1 for a summary) and there are currently moves to try and develop an OECD guideline for the *in vivo* Comet assay (www.ukems.org).

Table 4.1 Summary of the major reviews of the Comet assay

Authors and year	Main emphasis of review
(McKelvey Martin *et al.*, 1993)	Development of the alkaline version, technical variations, agents tested to date, analysis of DNA breakage, potential future usage
(Fairbairn *et al.*, 1995)	Principles of DNA strand breakage detection, neutral vs. alkaline methods, summary of technical details, applications, forms of DNA damage detectable by the assay
(Klaude *et al.*, 1996)	Technical considerations including neutral vs. alkaline methods, mechanisms of DNA breakage
(Collins *et al.*, 1997)	Range of applications, underlying principles, use of repair enzymes to increase sensitivity and specificity, kinetics of cellular repair
(Cotelle & Ferard, 1999)	Applications in Generic ecotoxicology
(Tice *et al.*, 2000)	Summary of International Workshop on Genotoxicity Test Procedures effort guidance for in vitro and in vivo use. Advantages of the technique, methodology, in vitro and in vivo specific considerations, future validation work
(Hartmann *et al.*, 2001)	Summary of screening of 250 drug candidates and the influence of cytotoxicity
(Hartmann *et al.*, 2003)	Guidance for in vivo methodology including discussion of cytotoxicity
(Duez *et al.*, 2003)	Statistics of the assay and how to approach data analysis
(Collins, 2004)	Methodologies including less common variants, apoptosis, image analysis and scoring, common applications, DNA repair and practical tips for successful and relevant cometing.

The comet assay gives details on the type of DNA problem and is quick easy and cheap (comparatively) to carry out.

4.1.3 Current studies on the toxicity of contaminated soil using the comet assay

The comet assay has been utilised in environmental genotoxicology studies (generally reviewed by Cotelle & Ferard, 1999) including investigations into the effects of soil contaminants. *In vivo* investigations using several earthworms species have examined the effects of specific soil contaminants spiked into soil including nickel chloride (Reinecke & Reinecke, 2004); pesticides (Zang *et al.*, 2000); and soil samples contaminated with aromatics (Verschaeve & Gilles, 1995) and PAH's (Salagovic *et al.*, 1996). Two studies (both using *in vivo* assessment) which combine biomonitoring and soil toxicity studies are those of Billeret *et al.* (2000) and Yanez *et al.* (2003). The former used rats exposed to contaminated soil to examine the genotoxicity of a highly polluted soil from a former coke plant; the contaminated soil was diluted with sand and rat lymphocytes assessed using the comet assay. The response was dose dependant showing the comet assay to be a sensitive biomarker. The study by Yanez *et al.* (2003) examined the genotoxic effect of lead and arsenic contaminated soils to children living in a mining area. Compared to children living in a control area the comet assay showed increased DNA damage to lymphocytes of those living with the contaminated soil.

4.1.4 Toxicity evaluation using human cells in vitro

The use of humans in toxicity testing is regarded as unethical and as such, other methods need to be found. Use of human cell lines *in vitro* can act as a reasonable surrogate for humans. The selection of a relevant tissue type or cell line to be used to evaluate toxicity (whether cytotoxic or genotoxic) is a vital part of any risk assessment process.

HepG2 cells

Within the UK a range of cell lines are available from the ECACC (European Collection of Cell Cultures) and from these, the HepG2 line was selected for use in this work. HepG2 cells are known as a good model to use in toxicity work as they

synthesise and secrete many normal plasma proteins (Knowles *et al.*, 1980) and have (low but stable) metabolic capacity that is usually lost to *in vitro* cultures. It is the longest established human liver cell line (1979), is not suspected of being infected with hepatitis B virus and its origin is genuinely hepatic (and not the result of metastasis of cells from another tissue type).

The cell line was established and first described by Aden *et al.* (1979) Taken from liver biopsies of a well differentiated hepatocellular carcinoma of a 15 year old Caucasian male, the cells present with an epithelial cell shape and morphological characteristics compatible with liver parenchymal cells. Particularly important metabolic enzymes are the cytochrome (CYP) P450's: phase I and also several phase II enzymes – these enzymes play a crucial role in activation of genotoxic pro-carcinogens and detoxification during phase II reactions. HepG2's show 10-20% mixed function oxidase (MFO) activity compared to freshly isolated hepatocytes (Doostdar *et al.*, 1988) therefore represent hazards of genotoxins more accurately.

Hartmann *et al.* (2003) explicitly say that a tissue should only be evaluated if there is evidence of exposure (*in vivo*). As outlined in Chap. 3 (bioavailability), ingestion has been identified as making the greatest contribution to any potential contaminant exposure. Ingested soil passes through the gastrointestinal (GI) tract and its products are taken to the liver to be broken down further (or "activated"), stored, or distributed and taken up (bioavailable fraction/uptake). In addition to being the major organ involved in metabolism, the liver is also responsible for detoxification of drugs and any foreign substances. As the site that would therefore have most concentrated and prolonged contact with potential contaminants (in the case of this study – metals) and as such be most prone to chronic damage, liver cells were chosen as a model to represent the potential for toxicity to humans in the *in vitro* experiments. HepG2 cells may be described as currently the most useful surrogate that is available.

Current uses of HepG2 cells in other studies

A recent search of academic literature (Web of Knowledge) demonstrates the popularity of the cell line, with over 6400 papers published. For example, Dehn *et al.*, (2004) assessed whether HepG2 cells would mimic known *in vitro* and *in vivo*

mammalian cell responses to cadmium. Using a raft of cytotoxicity assays, the cells were shown to respond to increasing concentrations of cadmium and the authors concluded that the cell line was a useful *in vitro* model. Majer *et al.* (2004) tested the genotoxicity of cadmium and arsenic (along with 9 non-metal compounds) using the micronuclei (MN) assay and found that HepG2 cells showed a dose dependant effect. Exploring a slightly different theme, HepG2 cells were used as the vehicle for examining copper-induced changes in protein expression by Roelofsen *et al.*, (2004). HepG2's have been used extensively in genotoxicity testing of pesticides, mycotoxins, polycyclic aromatic hydrocarbons (PAH) and many other organic compounds (reviewed by Knasmuller *et al.*, 1998 and 2004). HepG2 cells have also been used to examine the induction of metallothioneins and heat shock protein (Hsp70) in response to exposure to excess zinc and copper (Urani *et al.*, 2001; 2003). Overall, HepG2 cells have been shown to be a sensitive and highly suitable cell line for toxicity testing. Much of research has also been carried out using HepG2's and the comet assay in combination. For example Duthie & Collins, (1997) used the comet assay to compare the sensitivity of HepG2, Caco-2, HeLa and GM1899A (all human cell lines) to hydrogen peroxide, Uhl *et al.*, (1999; 2000), investigated a wide range of environmental pro-mutagens and found the use of HepG2 cells with the comet assay highly suitable for studying genotoxic effects with high reproducibility.

4.1.5 Experimental aims

The results of the investigations into the bioavailability of Cu, Ni, Pb and Zn from the 16 Byker soils (see Chapter 3) showed that of the 4 metals scrutinised, only Cu and Zn were released into the soil extract solutions in relatively high amounts and therefore were selected for further investigation.

The aims of this study were to

1. Investigate the sensitivity of HepG2 cells to metals (Cu and Zn) at levels found to be extracted from Byker soils (environmentally relevant).
2. Investigate whether using human cells *in vitro* can be used to determine the actual toxicity of metal contaminated Byker soils.

4.2 MATERIALS AND METHODS

Figure 4.1 outlines how work was carried out to investigate whether the human cell line, HepG2's, could be used to assess the toxicity of metal contaminated soils. All work was carried out using good laboratory practice and aseptic technique (when appropriate).

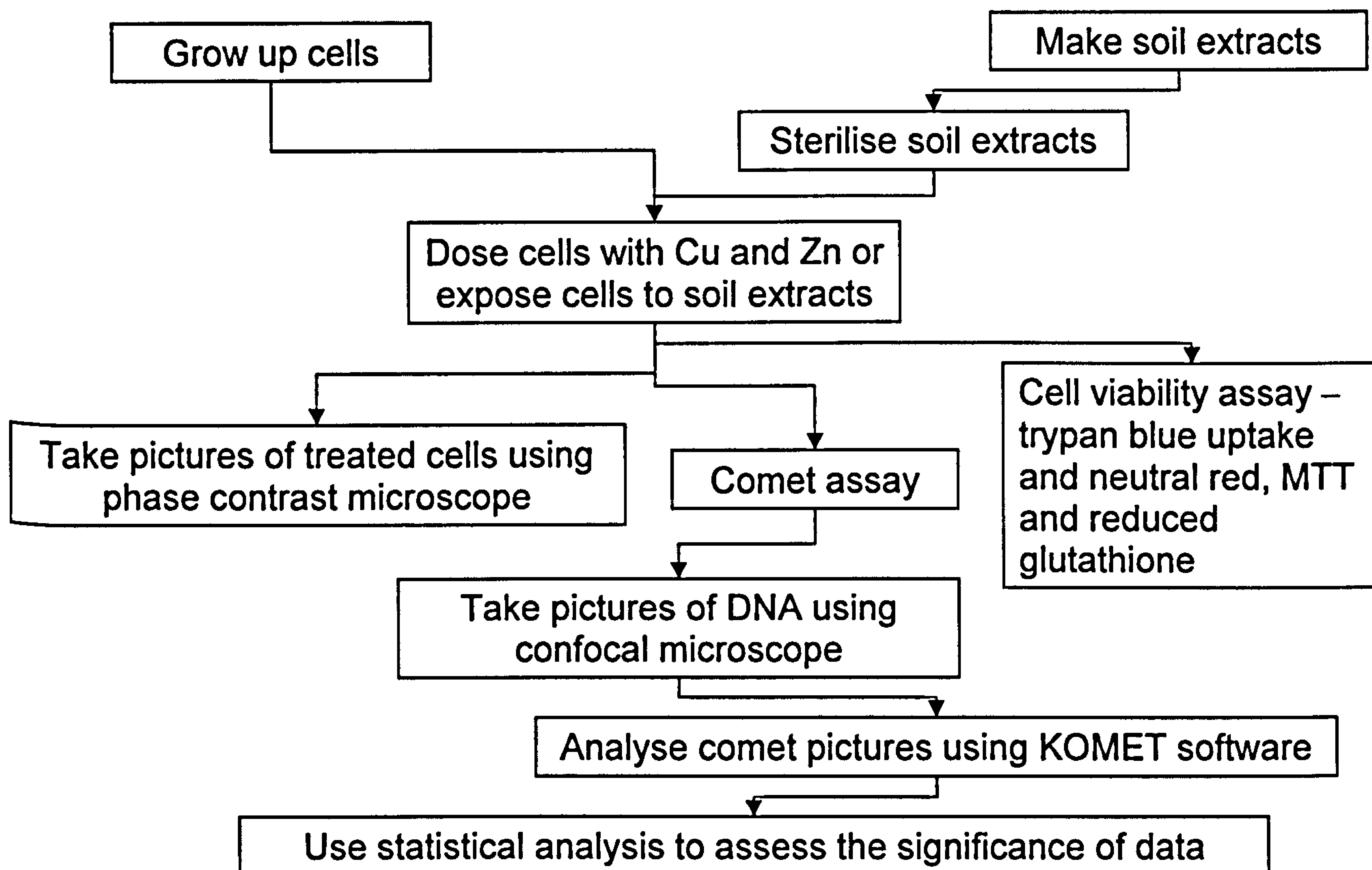


Figure 4.1 – Experimental plan to assess the potential of a novel *in vitro* human cell culture method to determine the toxicity of contaminated land

4.2.1 Culturing of HepG2 cells

The cells were obtained from the ECACC (www.ecacc.org.uk) and supplied frozen (-80°C) at approximately 100 passages and were stored in liquid nitrogen (-180°C) until required.

Cell culture medium

To grow and culture the HepG2 cells, Dubelccos minimum essential medium (DMEM) was used (Gibco) (also see 3.2.3). Additions required for growth and antimicrobial action were, per 500 ml DMEM; 50 ml foetal calf serum (FCS), 5 ml penicillin/streptomycin, 5 ml L-glutamine and 5 ml amphotericin B (Sigma and Invitrogen- concentrations as supplied). With additions the medium was kept at 4°C for a maximum of two weeks.

Revival of frozen cells

Cells were thawed swiftly (1-2 mins) and pipetted into pre-warmed (37°C) cell culture medium (5 ml per T25 flask) and placed in a sterile humidified incubator at 37°C with 95%air and 5%CO_2.

Culturing and Passage of cells

Cells were grown up and maintained in T25 cell culture flasks (vent cap/canted neck, treated, non-pyrogenic polystyrene) in 5 ml cell culture medium. The medium became exhausted every two-three days so was replaced with fresh medium three times a week. When 80-90% confluency was reached (usually in two weeks) or cells were required for assay purposes, the cells were passaged. Exhausted medium was discarded and cells were then washed with 10 ml phosphate buffered saline (PBS). The PBS was removed to waste and the wash repeated. A 5 ml EDTA (200 mg/L - Cambrex) wash was then used in order to stop "clumping" of cells. The EDTA was disposed of, replaced with 3 ml trypsin (0.05% w/v Cambrex) and then the cells were incubated for a maximum of five minutes. When the cells detached from the growth surface an equal or greater volume of medium was added in order to neutralise the trypsin action, the solution made up to 10 ml and either sub-cultured into further flasks or seeded into 24-well test plates at a density of 1.5×10^5/ml.

Maintenance of cell stocks

Due to the risk of infection of cells or time gaps between usages, a frozen stock was maintained. Whilst the cells were at a low passage number a stock was created. Cells were trypsinised and then neutralised. The cell suspension was centrifuged at 400 x g for 5 mins, the supernatant discarded and the pellet then re-suspended in freezing medium (90% FCS and 10% DMSO) at a density of 2-4x 10^6 cells/ml. The solution was then aliquotted into 1ml cryovials which were then stored in a CryoBox™. The cells were then frozen slowly, (down approximately 1°C/min) in a -80°C freezer overnight and then transferred into liquid nitrogen.

4.2.2 Preparation and sterilisation of DMEM/soil extract solutions before exposure to HepG2 cells

Extracts were prepared as described in section 3.2.1 (soil/water extract), section 3.2.2 (simulated gut extracts) and section 3.2.3 (DMEM/soil extract). Sterilisation of the solutions was necessary as preliminary experimentation showed that microbial growth occurred despite the addition of antibiotics to the cell culture medium, contaminating and out-competing the HepG2 cells.

Filtering

Soil/DMEM extracts were filtered through 0.2 μm sterile polysulphone filters (VWR) into gamma sterilised plastic centrifuge tubes. These filters are commonly used to sterilise tissue culture media that cannot be heat or pressure sterilised due to protein or sugar constituents. In this instance the function of filter sterilisation was to remove soil particulate matter and soil microbes prior to exposure to HepG2 cells.

Steaming

Soil/DMEM extracts were filtered (as described above) into autoclave sterilised, heat resistance plastic tubes which were then plugged with cotton wool. The tubes were stored in a large glass beaker and subjected to steam treatment for 1 hr followed by 23 hrs at 37°C. This cycle was repeated 3 times in order to

tyndalise the solutions and eradicate any contamination without destroying the constituents of DMEM essential for cell growth. Samples were then filtered again before use.

Gamma (γ) irradiation

Soil/DMEM extracts were prepared and filtered (as described above) into 15 ml tubes. A Gamma cell 1000 Elite (Nordion International Inc) with a Cs137 source, γ-irradiating at 3.27 Grays/min, was used. A cycle of 3.33 kGy (approx 17 hrs) at room temperature was completed overnight and the sample left for approximately 36 hours at room temperature. This routine was repeated three times until a 10 kGy total exposure had been reached. Samples were then filtered again.

4.2.3 Exposure and dosing of HepG2's with Cu, Zn, soil and soil extracts

In order to establish whether HepG2 cells were suitable as toxicity indicators for metal contaminated soils, it was necessary to verify that the cells were sensitive to metals at environmentally relevant levels (based on data in 3.3.3 and Table 3.9). Of the 4 metals in soils investigated in Chap.3, only Cu (0-14 mg/L) and Zn (0-1.5 mg/L) were shown to be extractable. This study therefore initially examined the sensitivity of HepG2 cells to Cu and Zn over a range of doses (0-100 mg/L) assessing the toxic responses (both cytotoxic and genotoxic). All assays were performed in triplicate using cells from increasing passages. The methods employed were then used to assess the toxicity (again both cytotoxic and genotoxic) of both the contaminated soils directly and the soil extracts created in Chap.3. Again, replication in triplicate of all assays was performed (where a result was produced).

Dosing with copper

HepG2 cells were seeded into 24-well disposable test plates (1 ml/well) and maintained at 37°C and 95% air and 5% CO_2 until 80-90% confluent. Cell culture medium was discarded, the cells rinsed with PBS and 1 ml DMEM,

without FCS, was added to each well. N.B. FCS is still a largely undefined component of cell culture medium and whilst necessary for growth may hinder dosing experiments by complexing the pollutants in the solution being tested. Copper standard solution ($CuNO_3.3H_20$- BDH) at 1000 mg/L was diluted for each condition. Per experiment, control wells were left without metal addition. The HepG2 cells were then exposed to a range of concentrations of copper (shown in Table 4.2) from 0-100 mg/L. Cells were then incubated with treatments for 24 hrs at 37°C and 95% air and 5% CO_2. This experiment was performed in triplicate.

Table 4.2 Concentrations of copper used to dose HepG2 cells

Concentration in mg/L	Concentration in µM
0.1	1.5
1	15.7
10	157.3
50	786.8
100	1573.6

shown in both mg/L and µM, atomic weight of copper – 63.546

The academic literature that describes *in vitro* dosing with metals gives data in a mixture of formats, some in weight per volume (e.g. mg/ml see Urani *et al.*, 2001) and some in moles (e.g. µM, see Roelofsen *et al.*, 2004). As the data generated in previous chapters is available in a weight/weight and weight/volume format, for the sake of consistency and ease of data interpretation it was decided to continue with this format and show data as weight/volume. The conversion from weight/volume to moles is also shown in Table 4.2 for Cu and Table 4.3 for Zn.

Dosing with zinc

A similar protocol as was used with Cu dosing was applied. Zinc standard solution ($Zn(NO_3)_2 \cdot 4H_2O$-BDH), 1000 mg/L, was appropriately diluted per parameter. The range of concentrations used was, again, 0-100 mg/L and the exposure time was 24 hrs. This experiment was also performed in triplicate. The conversion from weight/volume to moles is shown in Table 4.3.

Table 4.3 Concentrations of zinc used to dose HepG2 cells

Concentration in mg/L	Concentration in µM
0.1	1.5
1	15.3
10	152.9
50	764.7
100	1529.5

shown in both mg/L and µM, atomic weight of zinc – 65.38

Direct exposure of cells to contaminated soil

Cells were seeded into 24-well plates and grown until 80-90% confluent. Depleted medium was discarded and replaced with 1ml pre-warmed fresh DMEM (no FCS). 0.1 g of soil (all 16 soils described in Table 3.2 were used) was added to each well of a 24 well plate. 2 wells used as a control – i.e. no soil. This was incubated at 37°C and 95% air and 5% CO_2 for 1 hr. Waste medium was removed and cells were trypsinised and then neutralised. To separate and remove remaining soil waste from the cells, the resulting liquid was layered onto 5 ml Lymphoprep™ (a solution routinely used to separate blood cells components using a density gradient) and spun for 20 mins at 1200 x g. The band of cells produced at the Lymphoprep™/medium interface was pipetted from the Lymphoprep™ solution and added onto 5 ml fresh Lymphoprep™ and the centrifugation repeated until only cells remained.

Exposure of cells to non sterile soil extracts

Cells were seeded into 24-well plates and grown until 80-90% confluent. Cell culture medium was discarded and replaced with 1 ml of the appropriate soil extract (water, RIVM, BGS and DMEM extracts were used, see 3.2.1, 3.2.2 and 3.2.3). 2 wells per plate were kept as controls i.e. cells with fresh cell culture medium. Cells were then incubated for 24 hrs at 37°C and 95% air and 5% CO_2.

4.2.4 Image capture using Phase Contrast microscopy

Images of cells dosed with Cu or Zn for 24 hrs or exposed to soil extracts for 24 hrs were captured by a Leica DM IRB at x 20 and x 40 magnification and recorded using Leica QWIN software.

4.2.5 Cell viability assays

Subsequent to 24 hr dosing or exposure to Cu, Zn, soil and soil extracts, each cell dose was subjected to a battery of toxicity assays including both cytotoxicity (i.e. cell viability) and genotoxicity tests. Detailed below are the techniques that were used to assess a range of cell viability parameters, working on the premise that reduced viability was equivalent to cytotoxicity.

The cytotoxicity assays performed examine different aspects of cell viability; both the trypan blue and neutral red assays look at cell membrane integrity (although the trypan blue method is a much more crude and error prone measure), the MTT assay is a measure of mitochondrial (i.e. metabolic) capability and the reduced glutathione assay indicates the oxidative stress status of the cells.

Trypan blue dye exclusion assay

Trypsin harvested cells were mixed 1:1 with trypan blue stain (0.4% w/v, Biowhittaker) and pipetted into a haemocytometer counting chamber and counted at x 40 magnification on a Jeneval bench top light microscope. If membrane integrity had been compromised by metal or soil extract exposure (i.e. cells were dead/dying) then the cells absorbed the dye and appeared blue. Conversely cells with undamaged membranes exclude the colour. Less than 80% viability is indicative of troubled cell population (Philips, 1973). The data gathered (total number of cells) was also used as an estimation of cell number. The volume of the counted square is 0.1 mm^3 or 0.1 μl. Use of this figure enabled a rough cell number to be inferred.

Neutral red uptake assay

Neutral red A is a supravital dye that is taken up by lysosomes in living cells. The method used was adapted from Hunt *et al.*, (1987) and Cusack *et al.*, (2005). Cells were grown and incubated with treatments (metal or soil extract) as usual for 24 hrs in 24-well plates. The medium was discarded and replaced with 1 ml DMEM plus 0.01 ml/L Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) solution (BDH). Cells were then incubated for 3 hrs at 37°C and 95% air and 5% CO_2. This medium was discarded and cells were then solubilised with 1 ml glacial acetic acid/ethanol solution (1:100 v/v). 200 µl of each treatment was transferred into a flat bottomed clear 96-well plate and absorbance measured immediately at 570 nm with a Thermo Labsystems Multiskan Spectrum plate reader. Results were expressed as a percentage of the control (100%) with +/- 20% showing a toxic effect.

MTT assay

The assay is based on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide) to formazan by succinate dehydrogenase; the formazan product is then quantified. The method used was adapted from Mossman (1983) and Cusack *et al.* (2005). Cells were grown and incubated with treatments (metal or soil extract) as usual for 24 hrs in 24-well plates. The medium was then discarded and replaced with 1 ml DMEM plus 0.6 mg/ml MTT (Sigma). The cells were incubated for 4 hrs at 37°C and 95%air and 5%CO_2. This medium was removed to waste and the plates were left at minus 20°C overnight to induce cell rupture. After this time 1 ml of propanol was added to each well to solubilise cells and dye crystals. Once all the dye had dissolved, 200 µl of each treatment was transferred into a flat bottomed clear 96-well plate and absorbance measured immediately at 570 nm with a Thermo Labsystems Multiskan Spectrum plate reader. Results were expressed as a percentage of the control (100%) with +/- 20% showing a toxic effect.

Reduced glutathione (GSH) depletion assay

Cells were grown and incubated with treatments (metal or soil extract) as usual for 24 hrs in 24-well plates. The medium was discarded and the cells briefly washed with PBS. 0.5 ml trypsin was added to each well plus 100 µl monobromobimane (MBBr – 3 mM: 25 mg powder dissolved completely in 100 µl acetonitrile and then diluted in 30.4 ml 50 mM aqueous n-ethylmorpholine). Glutathione standards (0, 5, 10, 20 and 40 nM) were made up in 1.5 ml microcentrifuge tubes and 100 µl MBBr was added to each (both supplied by Sigma). Samples and glutathione standards were then incubated at 37°C and 95% air and 5% CO_2 in the dark for 30 mins. All samples were then removed to 1.5 ml microcentrifuge tubes and 10 µl tricholoracetic acid (TCA) was added. Samples and standards were then centrifuged at 13,000 x g for 5 mins to remove cell debris. The supernatant was carefully removed to HPLC vials which were sealed and analysed immediately.

HPLC analysis of reduced glutathione

The protocol followed was one devised and frequently used by the Toxicology group of Newcastle University (Gage, 2005). The GSH content of the cells in incubation was quantified by comparison to the GSH standard curve (0-40 nM GSH). A Kontron system with a 420 pump, 425 gradient former, 360 autosampler and SFM25 fluorescence detector was used. The system was controlled by a Kontron Data System 450, software version 3.30 and the column used was a 150 mm x 4.6 mm Hypersil 3ODS with a Waters C18 guard column and a flow rate of 1.3 ml/min. The mobile phases used were: A – 100% acetonitrile, B-50/50 acetonitrile/H_2O, C- 10% v/v acetonitrile in 0.25% v/v acetic acid (pH3.7). The fluorescence detector had an excitation wavelength of 390 nm, an emission wavelength of 477 nm and response time was 0.5. Total run time was 22 mins with mobile phase times shown in table 4.4. Using these conditions GSH eluted at 9.2 mins. A typical HPLC trace can be seen in figure 4.2

Table 4.4 GSH assay HPLC mobile phase conditions

Time in mins	ACN % B	ACN % C
0	0	100
4.5	0	100
5	35	65
11	35	65
12	0	100
15	0	100

The concentration of the GSH/incubation (in nmol GSH) was divided by the protein concentration of the cells to give results in nmol GSH/ mg protein.

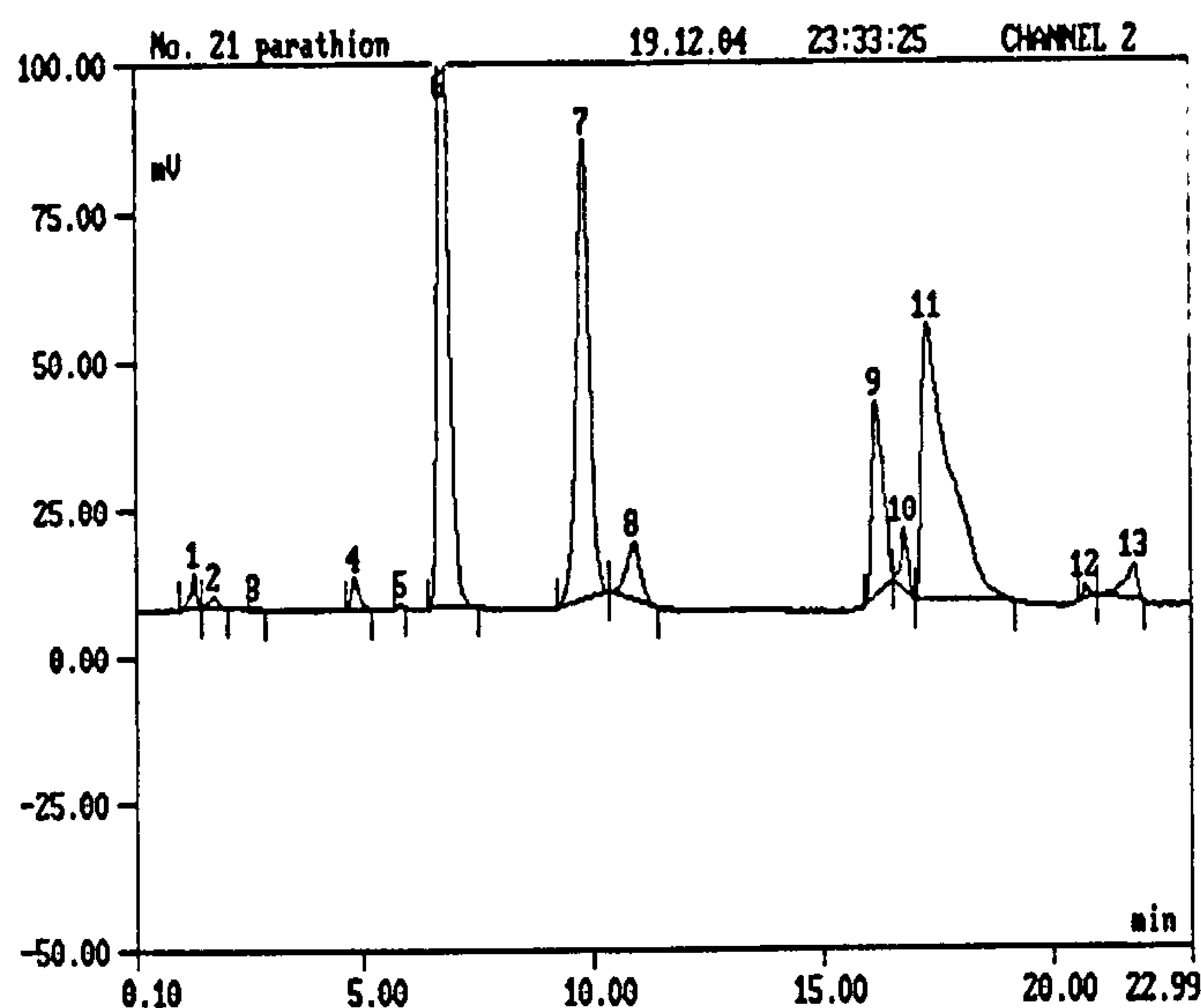


Figure 4.2. A typical HPLC trace for identifying GSH levels.
Peak 6=excess MMBr (dye) and peak 7=MMBr-GSH (dye+glutathione)

Analysis of the protein content of HepG2 cells

The procedure was adapted from the method described in Smith *et al.* (1985), and used to estimate the amount of protein (i.e. cells) present. Cells were grown up and incubated with treatments as usual for 24 hrs in 24-well plates. The medium was then removed to waste and 0.5 ml of 0.5 M NaOH was added to solubilise the cells. This solution was removed to 1.5 ml microfuge tubes and stored at 4°C until analysed.

Protein standards were prepared (BSA, 200 mg/ml, Sigma) to final concentrations of 0, 100, 200, 300, 400, 500, 600 and 700 μg/ml. These were vortexed to mix thoroughly.

Reagent was (freshly) prepared using bicinchoninic acid (BCA) assay solution which was mixed with 4% (w/v) copper sulphate solution at a ratio of 50:1 v/v. 25 μl of samples and standards was then added to a flat bottomed clear 96-well assay plate and 225 μl of BCA/copper sulphate solution was added to each well. The plate was incubated for 1 hr at 37°C and 95% air and 5% CO_2 and then read at 562 nm with a Thermo Labsystems Multiskan Spectrum plate reader.

4.2.6 - Comet assay

As outlined in 1.8 and 4.1.2, the comet assay is a technique that can be used to quantify DNA strand breaks in any eukaryotic cell population. HepG2 cells were exposed to Cu, Zn, soils or the appropriate soil extracts and then subjected to the comet assay (see below).

Materials

Low melting point agarose (LMPA) MacroSieve LMPA (Flowgen) can be used for separation of compounds >1kb. For a 2% solution, 1 g was dissolved in 50 ml PBS. For a 1% solution, 25 ml 2% solution was added to 25 ml PBS.

Lysis buffer 73.05 g of sodium chloride, 100 ml 0.5 M EDTA (pH 8), 16.6 ml of 30% Sarkosyl solution and 5 ml 1 M Tris base (pH 10) was made up to 400 ml with deionised water and mixed with a magnetic stirrer for 1 hr. 50 ml DMSO and 5 ml Triton X-100 were added just before use.

Alkali Buffer 24 g sodium hydroxide and 4 ml 0.5 M EDTA (pH 8) was made up to 2 L with deionised water.

Neutralising Buffer 60.57 g Tris base was made up to 1 L with deionised water.

Comet assay experimental procedure

Superfrost® Plus microscope slides were coated with 100 µl of 1% LMPA to aid adhesiveness and left to dry for 1 hr. Slides were then placed on a pre-cooled metal tray (resting on an ice bed). LMPA (170 µl of 1%) was pipetted onto a slide, shaped using a glass coverslip and left to set. A second layer (100 µl 2% LMPA) was mixed with 100 µl cells and added to the slide. The third layer consisted of 100 µl 1% LPMA.

All buffer and electrophoresis stages were carried out in the dark and all solutions were pre-cooled on ice. Once dry and with the coverslip removed, slides were placed in lysis buffer and left for 1 hr. Slides were then gently removed (sliding out of the buffer so as not to lose the agarose sandwich) and rinsed in PBS for 15 mins. Slides were then moved to a horizontal electrophoresis tank (Pharmacia Gel Electrophoresis Apparatus GNA-200), filled with alkali buffer and cooled on an ice bed. The slides were placed in the tank (straight and lined up with each other, in order to prevent electrophoresis occurring at an angle) and incubated in the buffer for 30 mins. The slides were electrophoresed for 30 mins at 22 V and 500 mA using a Biorad Powerpac 200 and then placed in neutralising buffer for 15 mins, followed by PBS for a final 15 mins. The slides were then stored in a moist cool environment (never more than two days) until the DNA was stained just before image capture.

DNA Staining

SYBR® Gold nucleic acid gel stain (S11494, Molecular Probes) stock was diluted to make a 1x staining solution (fresh dilution on each occasion); 1 µl in 10 ml of buffer, TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5-8.0). Designed as a stain for single and double stranded DNA; SYBR® Gold was used for fluorescent enhancement of cells. Slides were saturated in the 1x solution and incubated for 10-40 mins to allow the stain to permeate the gel and "develop". The dye was chosen for use over the traditional DNA stain, ethidium bromide- due to increased sensitivity (>10-fold) and reduced cancer risk to the user.

4.2.7 Image capture using Confocal microscopy

A scanning laser confocal microscope (Leica TCS SP2 UV) using Leica Confocal Software (version 2.5 Build 1347) was used to capture images of cells. Settings used were 568 and -1; 488 laser; x 20 lens; zoom 1.5; 14 sections of 60 μm. 50 cells per condition (25 per slide) were recorded to confer statistical significance of results. This work was carried out in the Bio-imaging Unit, University of Newcastle upon Tyne with support from Dr Trevor Booth.

4.2.8 Image analysis (KOMET software)

Images of cells were analysed using KOMET version 4.0.2 (Kinetic Imaging Software). For each cell scored, a wide range of parameters are measured and recorded and as such, were accessible for analysis. Parameters available included % DNA in the head, % DNA in the tail, Olive Tail Moment (OTM), Tail length (in μm), tail extent moment and mode, mean, SD, inertia and skew of head, tail and comet.

4.2.9 Statistical analysis

All calculations for cytotoxicity assays were carried out using Microsoft Excel. MINITAB® 14 Statistical software for Windows was used to analyse OTM and % tail DNA (parameters most widely used). Both tail % DNA and OTM probability plots show data distribution to be non-Gaussian (Duez et al., 2003) precluding the use of parametric tests. The Mann-Whitney U (post-hoc) test (examines the equality of two population medians using the null hypothesis (H0) that increasing concentration of metal does not increase DNA damage) was used to show whether medians of different conditions were equal (null hypothesis) with $P<0.05$ considered to be significant.

4.3 RESULTS

4.3.1HepG2 cell response to exposure to Cu

Figures 4.3 shows images of the HepG2 cells exposed to 0 mg Cu/L (a) and 50 mg Cu/L (b) taken using a phase contrast microscope; these pictures illustrate the changes that can be induced in cell morphology when exposed to toxic levels of a chemical or compound. Further phase contrast images of dosed HepG2 cells (at x 20 and x 40 magnification) can be seen in Appendix 4.

a) b)

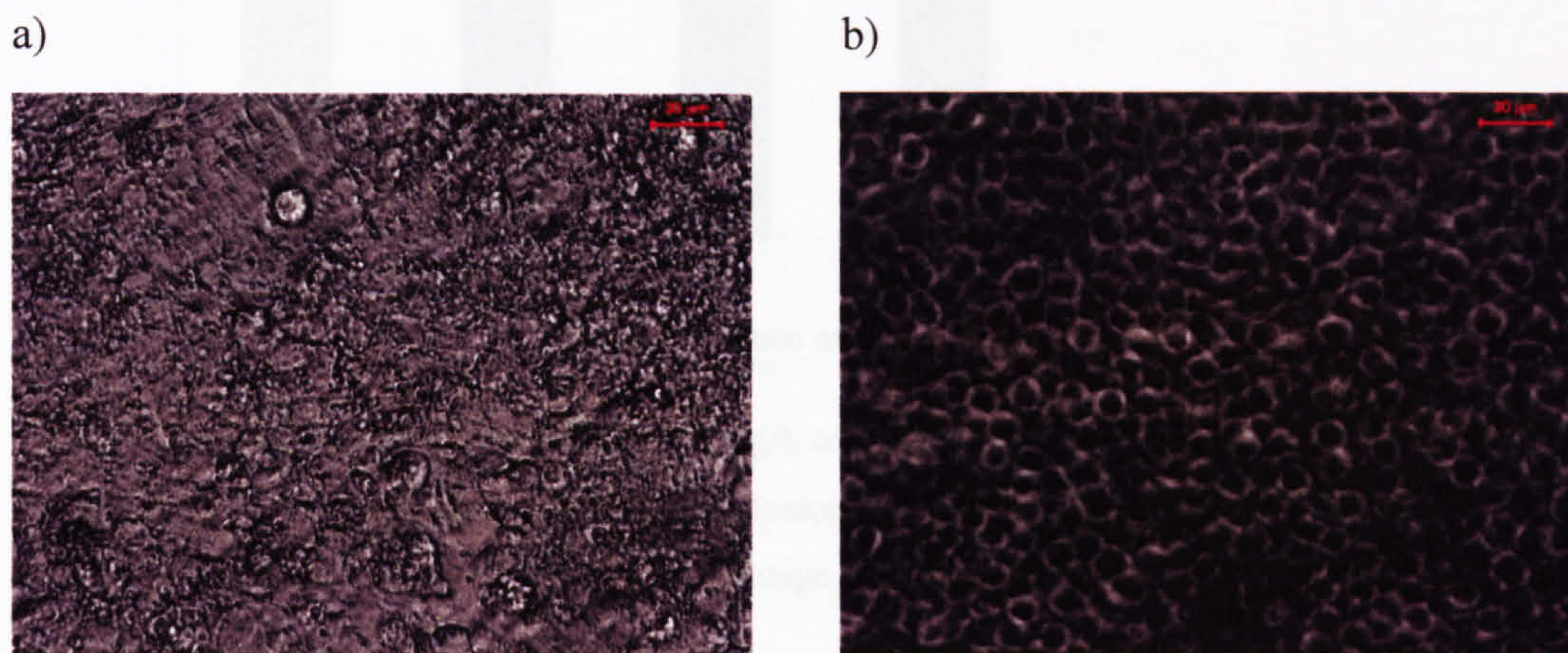


Figure 4.3 HepG2 cells exposed to 0 mg Cu/L (a) and 50 mg Cu/L (b)
X 20 magnification (red bar shows 30 μm scale)

Viability of HepG2 cells treated with increasing concentrations of Cu

The cytotoxicity assays performed examined different aspects of cell viability including cell membrane integrity, metabolic capability and the oxidative stress status. Both the trypan blue (Fig 4.4) and neutral red (Fig 4.5) assays evaluated cell membrane integrity (although the trypan blue is a much more crude and error prone measure).The trypan blue assay showed that at 0.1 and 1 mg Cu/L, the cell membrane integrity of the cell population was within the +/-20% range of the control. At 10 mg Cu/L the population of cells with viable cell membrane integrity was still within the acceptable range but with greater levels of standard error. At 50 and 100 mg Cu/L the cell population had <20% membrane integrity and therefore was essentially dead. The neutral red assay showed similar results to the trypan blue; at 0.1 and 1mg Cu/L, cells were within the +/-20% of the control range, 10 mg Cu/L the cell membrane integrity dropped to ~80% of the control and at 50 and 100 mg Cu/L the cells were in effect dead.

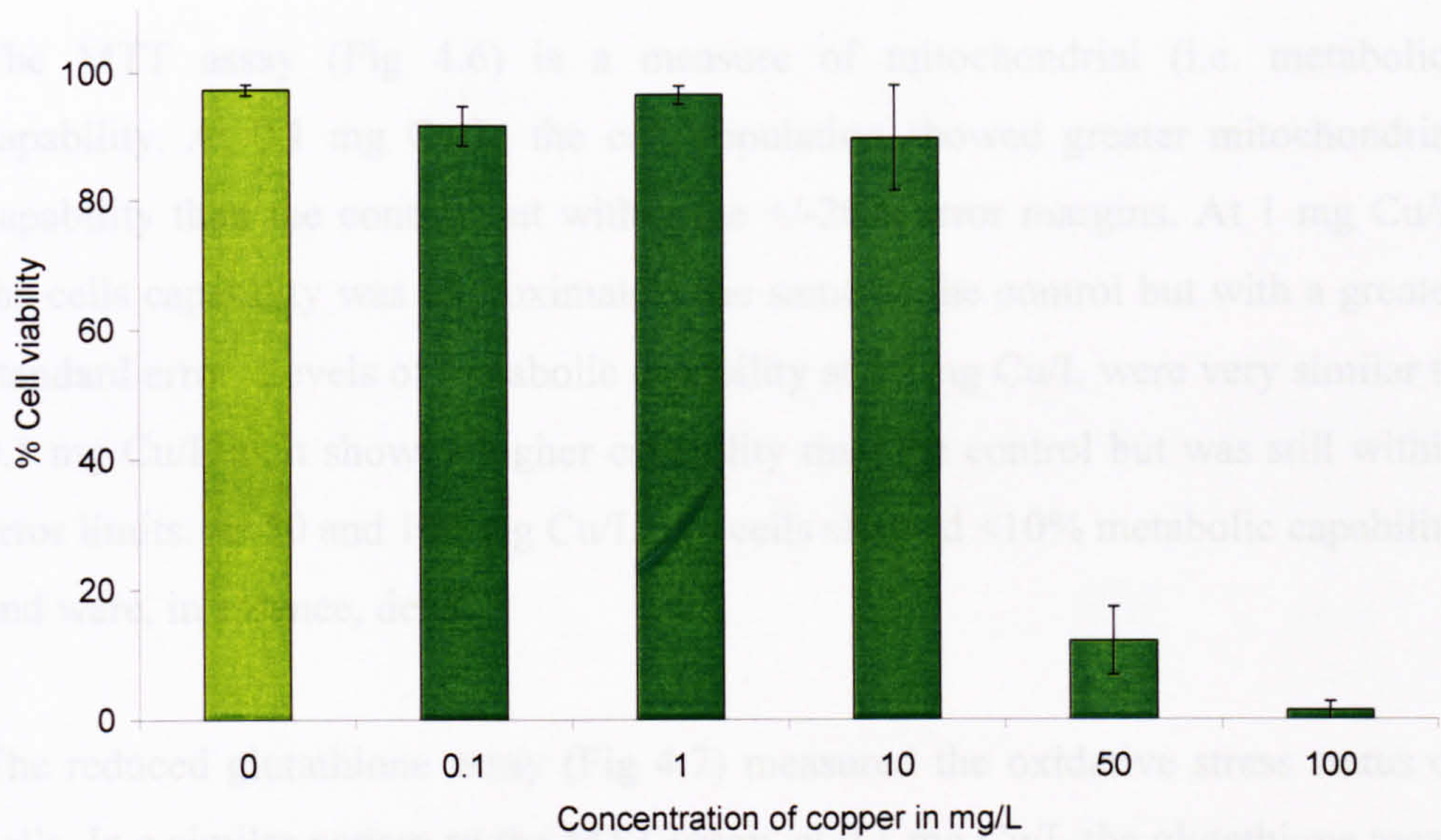


Figure 4.4 – HepG2 cells exposed to 0-100 mg/L of Cu for 24hrs; live/dead assessment using the Trypan blue dye exclusion cell viability assay.

Results are expressed as a percentage of the control +/-SEM (n=3)

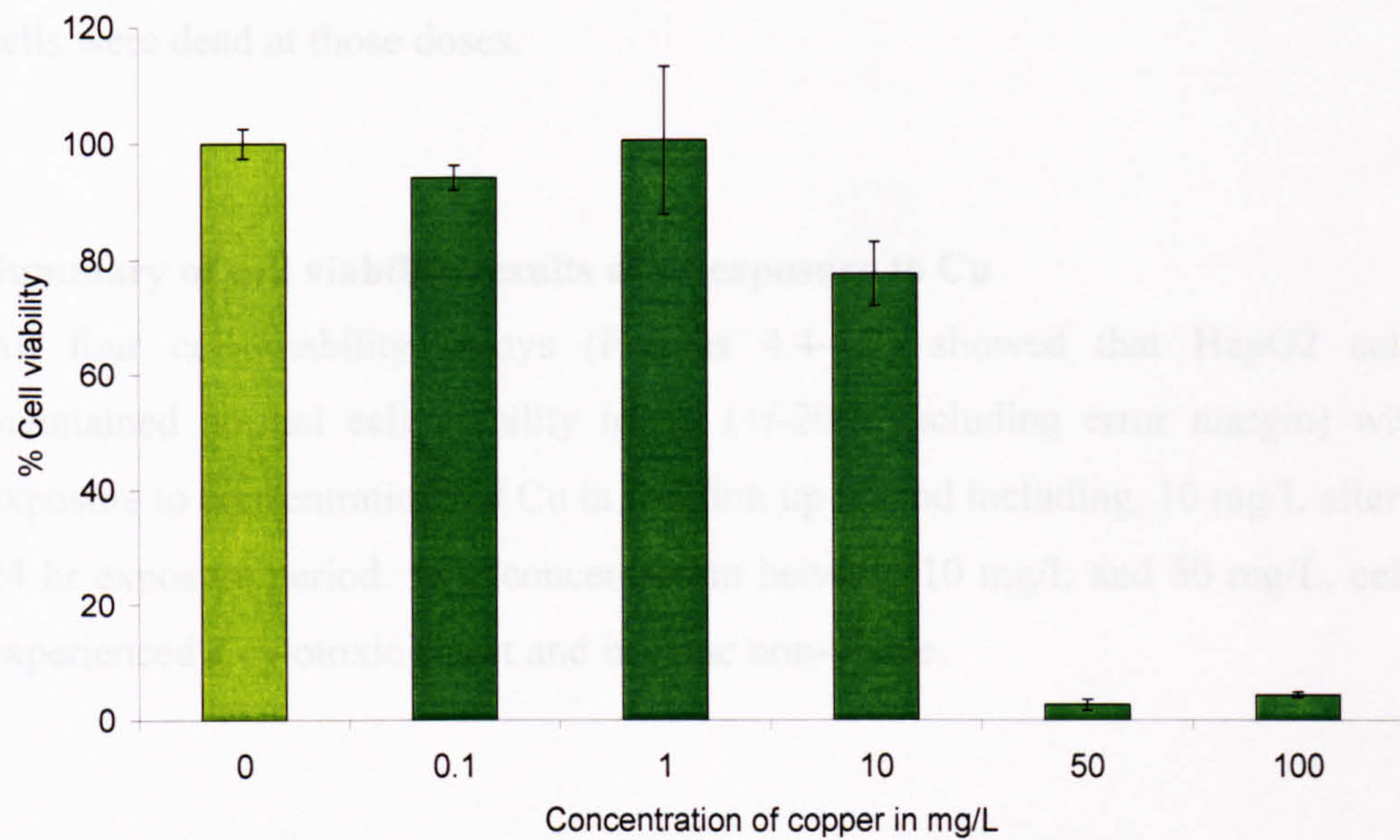


Figure 4.5 –HepG2 cells exposed to 0-100 mg/L Cu for 24hrs; live/dead assessment using the Neutral Red dye uptake cell viability assay.

Results are expressed as a percentage of the control +/-SEM (n=3)

The MTT assay (Fig 4.6) is a measure of mitochondrial (i.e. metabolic) capability. At 0.1 mg Cu/L, the cell population showed greater mitochondrial capability than the control but within the +/-20% error margins. At 1 mg Cu/L the cells capability was approximately the same as the control but with a greater standard error. Levels of metabolic capability at 10 mg Cu/L were very similar to 0.1 mg Cu/L as it showed higher capability than the control but was still within error limits. At 50 and 100 mg Cu/L, the cells showed <10% metabolic capability and were, in essence, dead.

The reduced glutathione assay (Fig 4.7) measured the oxidative stress status of cells. In a similar pattern to the MTT assay, at 0.1 mg Cu/L the glutathione assay showed levels above the control. At 1 mg Cu/L levels were comparable to the control and at 10 mg Cu/L levels were above the control and close to those of 0.1 mg Cu/L exposure. At 50 and 100 mg Cu/L no activity was seen at all i.e. the cells were dead at those doses.

Summary of cell viability results after exposure to Cu

All four cell viability assays (Figures 4.4-4.7) showed that HepG2 cells maintained normal cell viability levels (+/-20% including error margin) with exposure to concentrations of Cu in solution up to, and including, 10 mg/L after a 24 hr exposure period. At a concentration between 10 mg/L and 50 mg/L, cells experienced a cytotoxic effect and became non-viable.

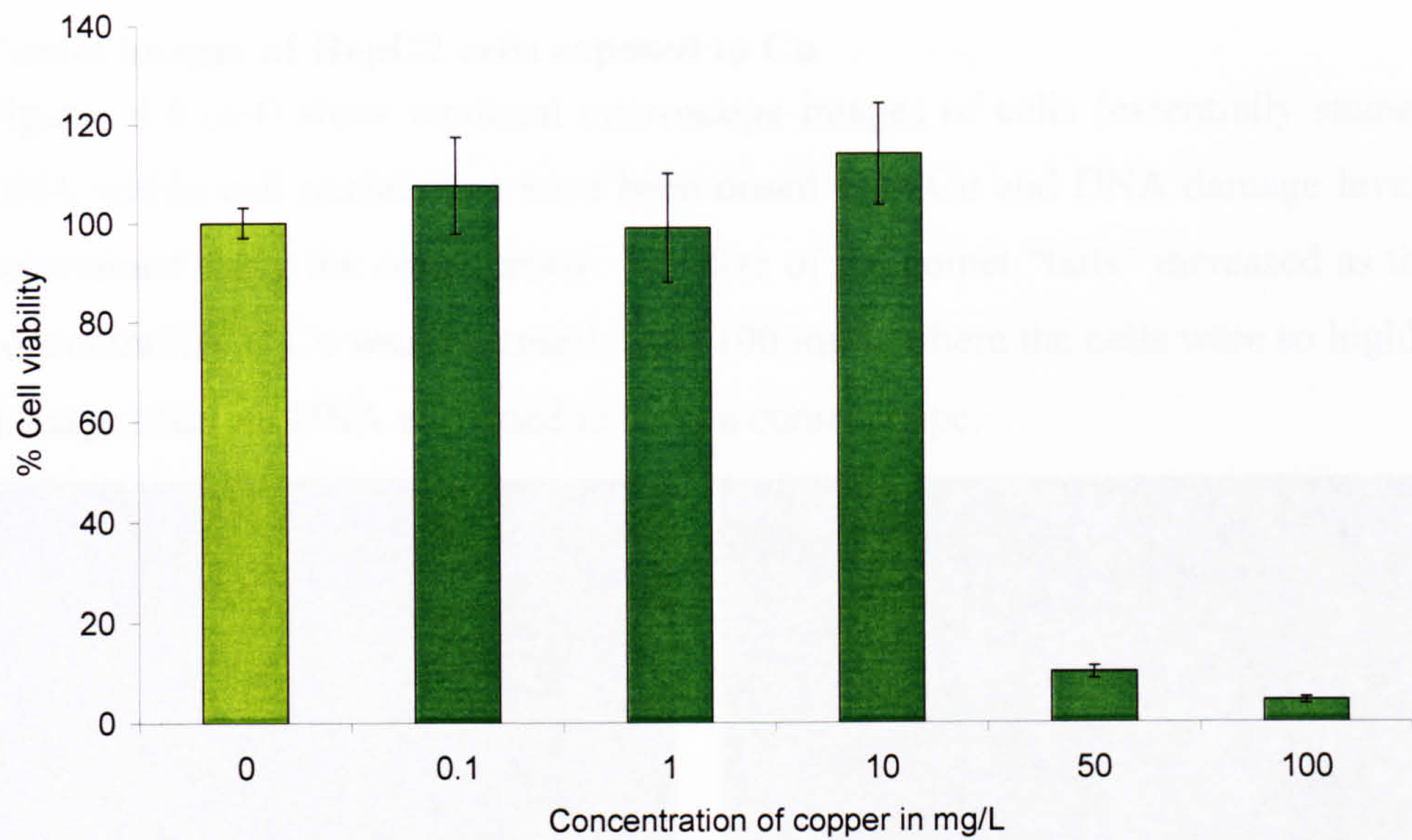


Figure 4.6 – HepG2 cells exposed to 0-100 mg/L of Cu for 24hrs; live/dead assessment using the MTT cell viability assay. Results are expressed as a percentage of the control +/-SEM (n=3)

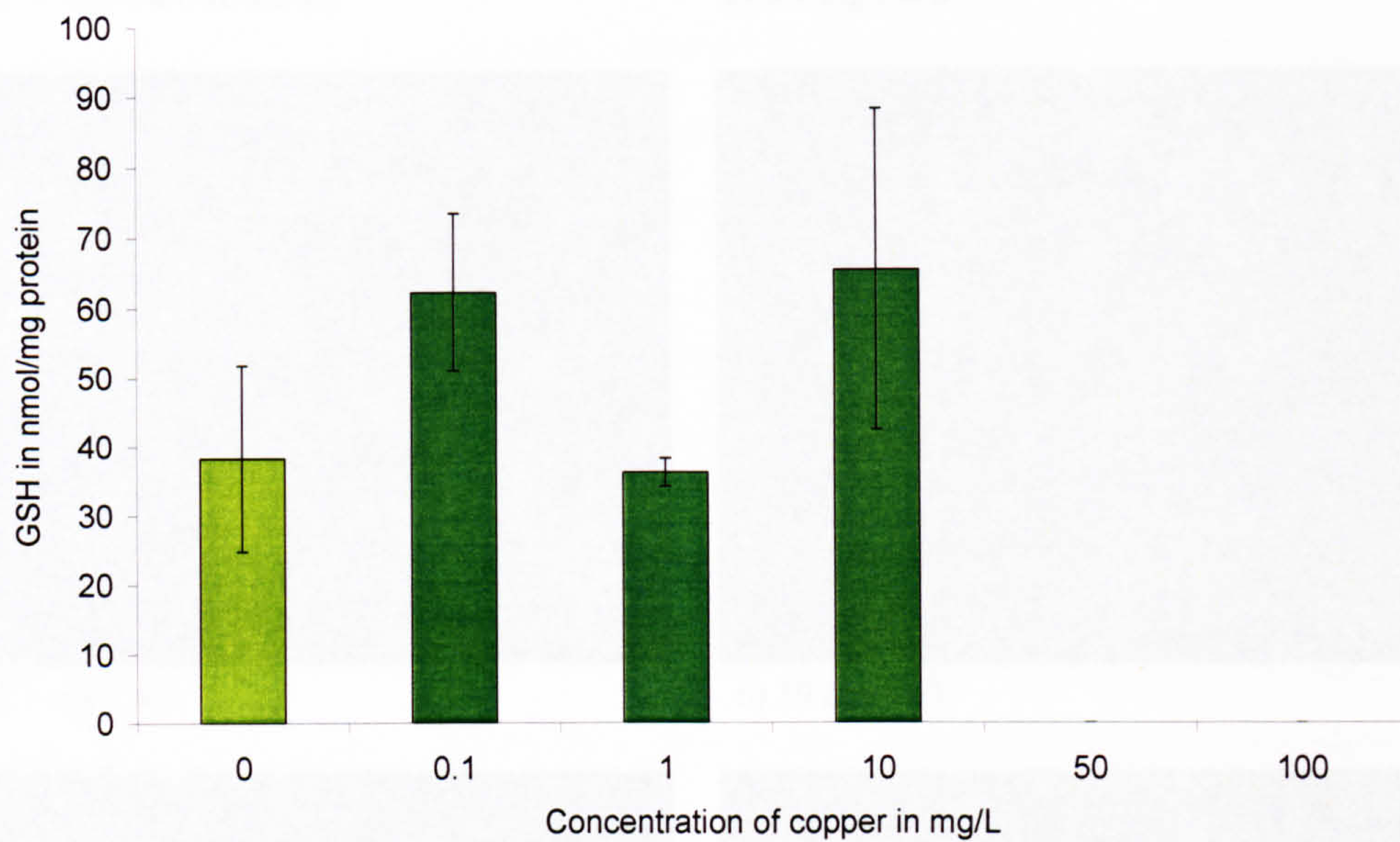
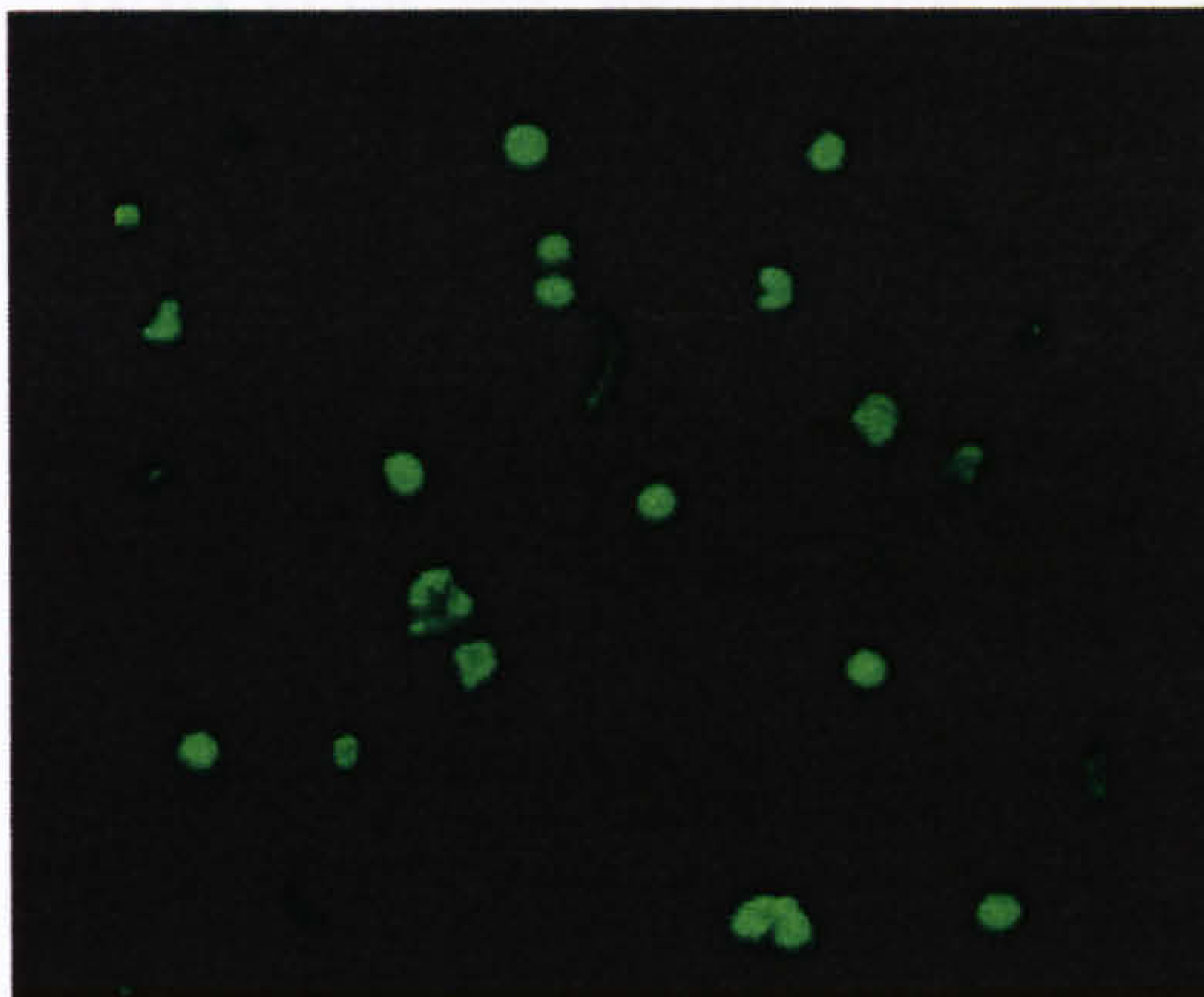


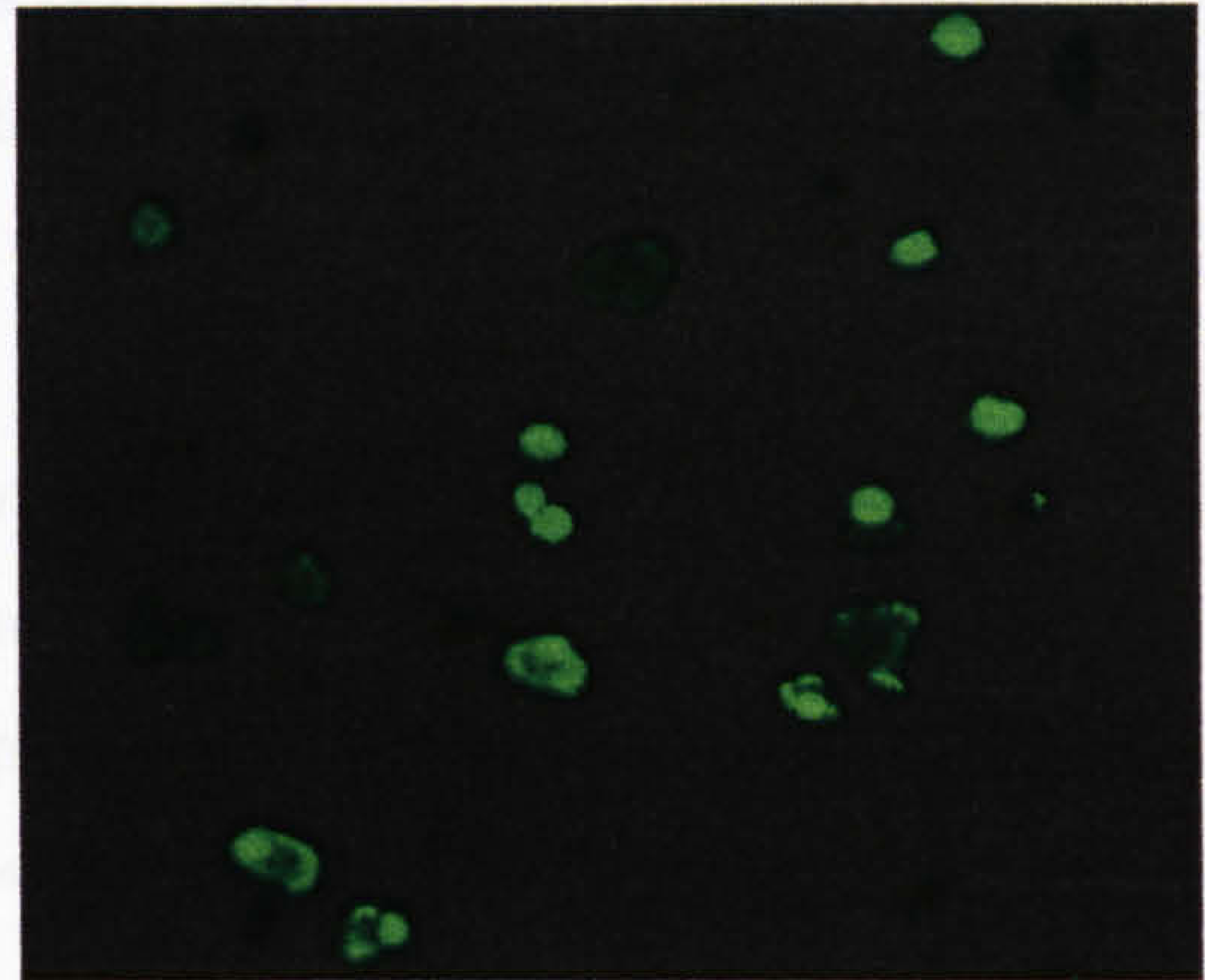
Figure 4.7 – HepG2 cells exposed to 0-100 mg/L of Cu for 24hrs; reduced glutathione determination. Results are expressed in nmol GSH/mg protein +/-SEM (n=3)

Comet images of HepG2 cells exposed to Cu

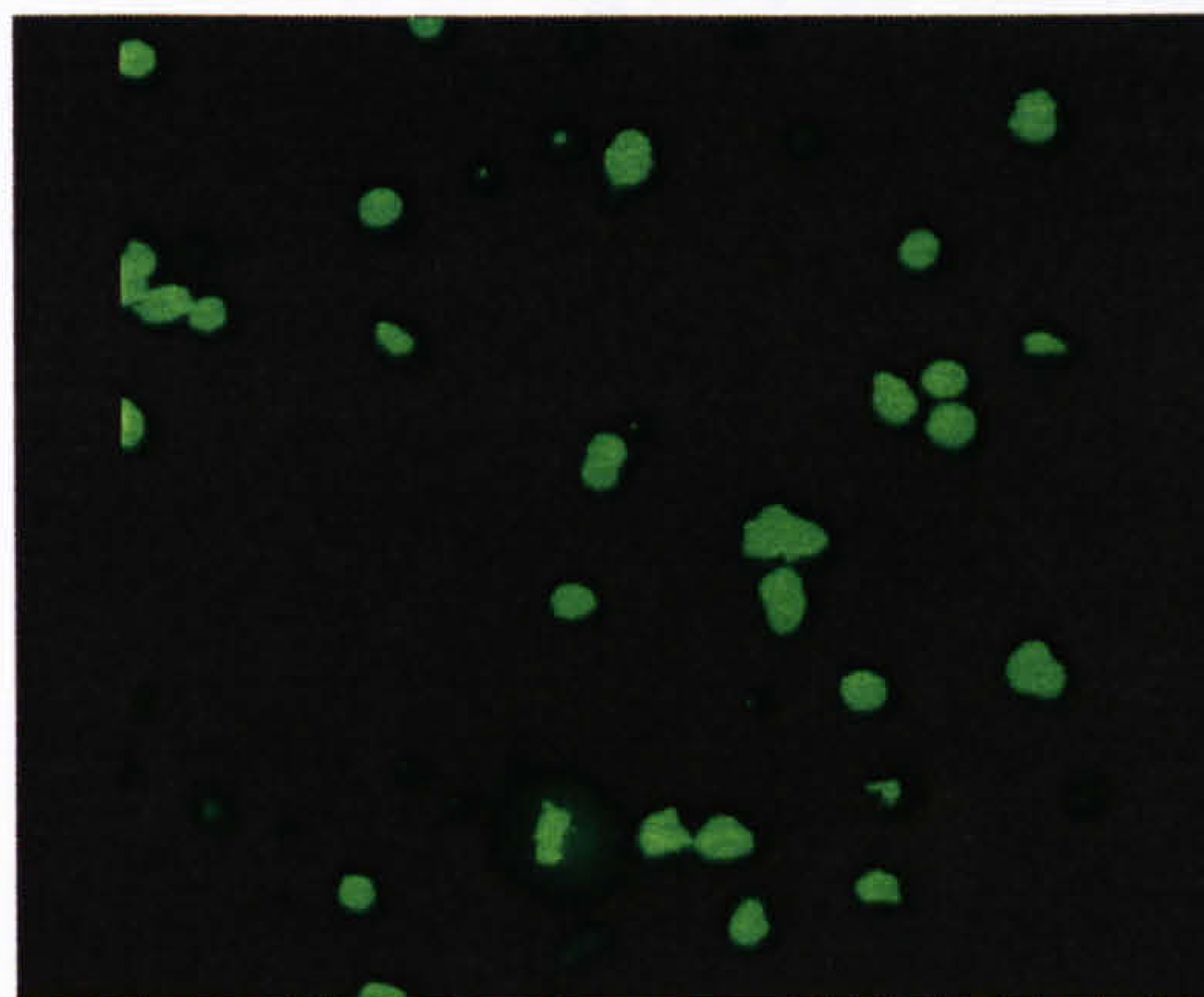
Figures 4.8 (a-f) show confocal microscope images of cells (essentially stained DNA within cell nuclei) that have been dosed with Cu and DNA damage levels determined using the comet assay. The size of the comet "tails" increased as the concentration of Cu was increased, until 100 mg/L where the cells were so highly damaged that no DNA remained to form a comet shape.



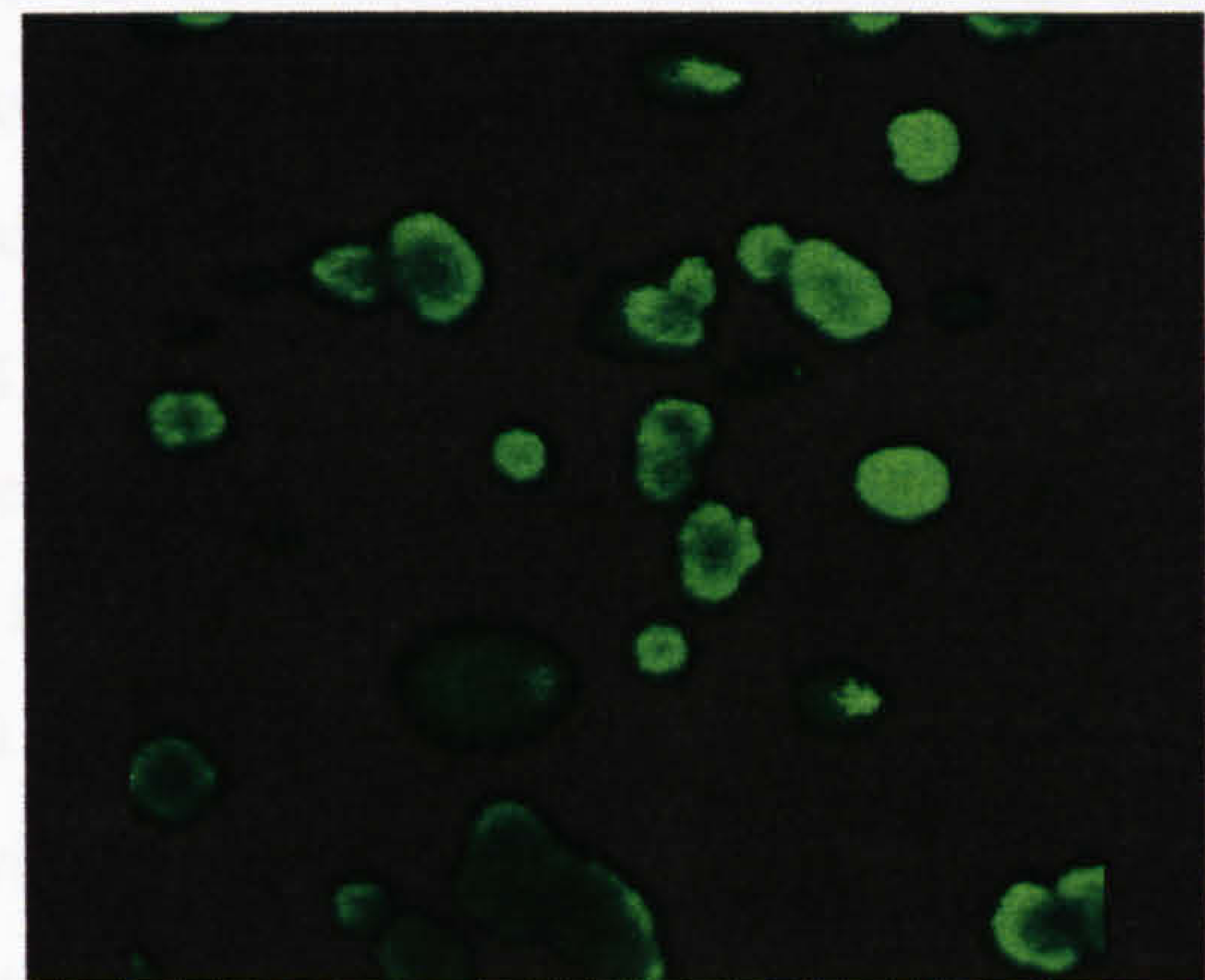
a) 0 mg Cu/L (control)



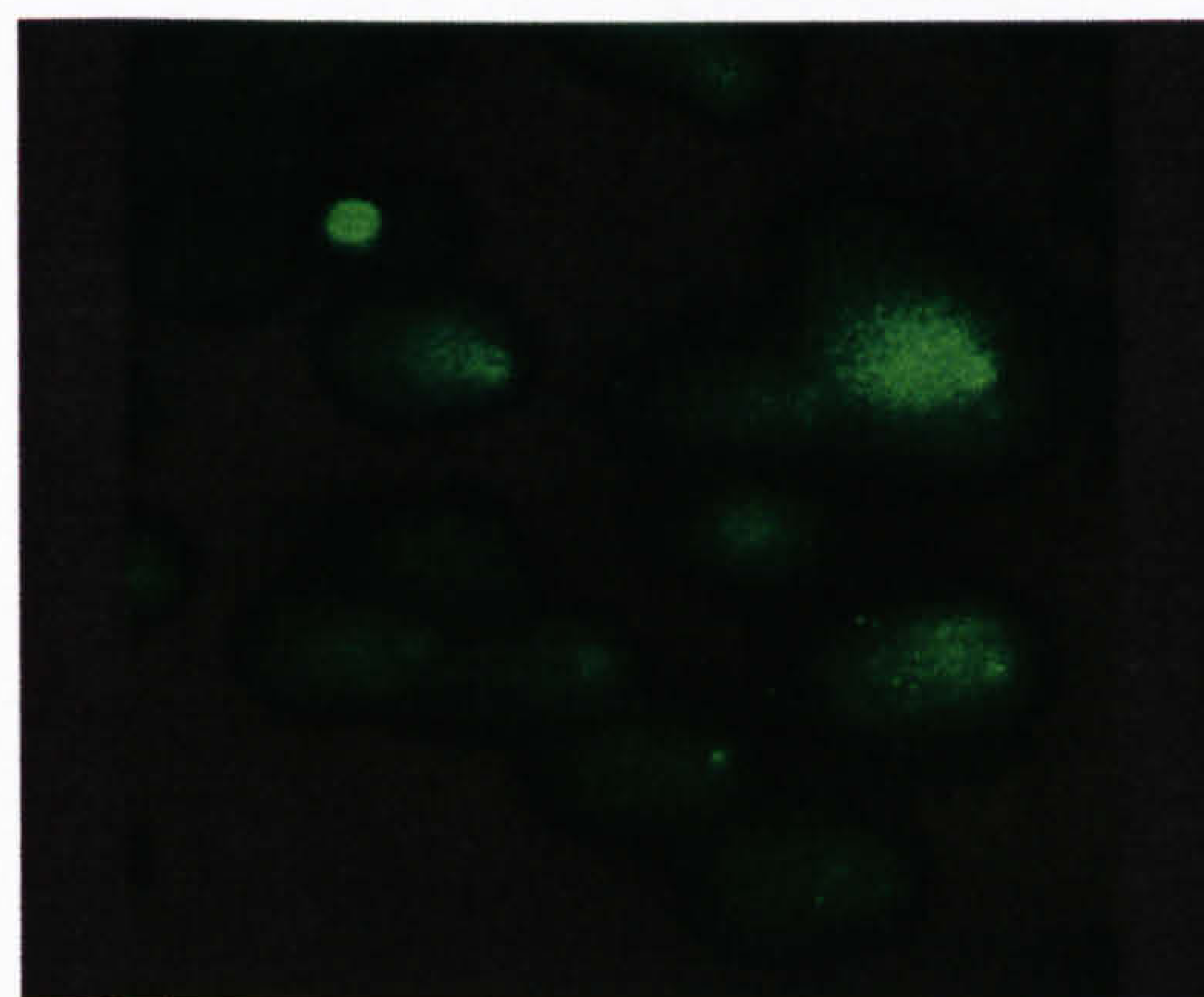
b) 0.1 mg Cu/L



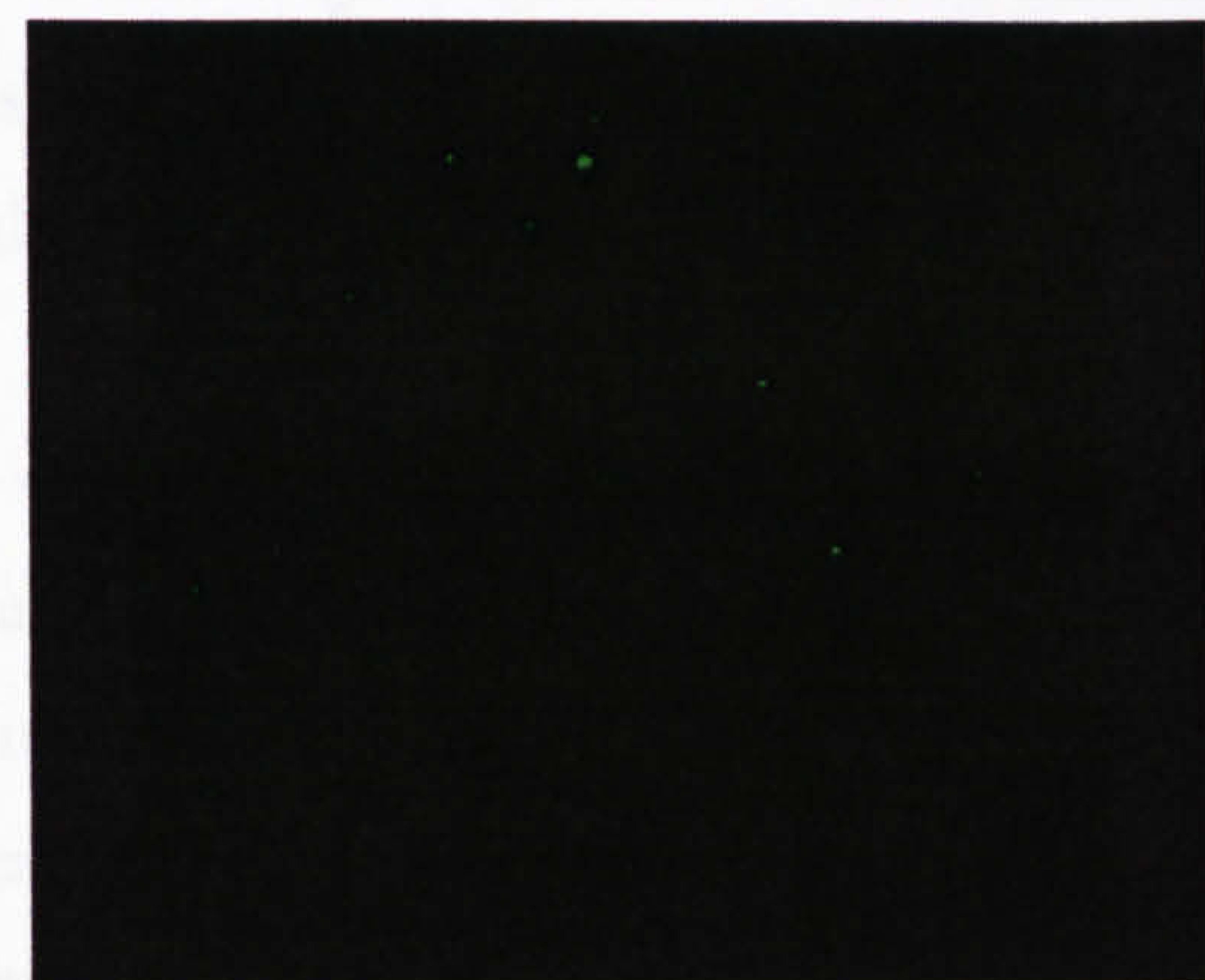
c) 1 mg Cu/L



d) 10 mg Cu/L



e) 50 mg Cu/L



f) 100 mg Cu/L

Figure 4.8 Confocal microscope images of HepG2 cells exposed to increasing concentrations of Cu. Direction of electrophoresis was right to left

DNA damage to HepG2 cells treated with increasing levels of Cu

Figures 4.9 to 4.12 show the increasing levels of DNA damage (as seen by the comet assay) that occur to HepG2 cells after exposure to increasing levels of Cu. Two parameters were examined; firstly the percentage tail DNA which measures the % of DNA in the comet tail compared to that in the comet head, i.e. the more DNA in the tail, the more the DNA has been damaged and unwound. Secondly the tail moment; this is defined as the product of the tail length and the percentage of total DNA in the tail.

Control cells (Fig 4.14 shows a box plot of % tail DNA) exposed to 0 mg/L Cu had 0-15% of DNA present in the comet tail (signifying normal levels of cell DNA damage). In comparison, after exposure to 0.1 mg/L Cu, the % of DNA in the comet tail increased significantly ($P<0.05$) to between 5 and 30%. At 1 mg/L Cu, the main range of tail % DNA (between 0 and 20%) was lower than when exposed to 0.1 mg Cu/L and whilst appearing quite similar to the control was still significantly ($P<0.05$) different with both the mean and median being higher. Further increases in Cu levels led to increasing amounts of DNA present in the comet tails with all levels of Cu causing levels of % DNA in the comet tail to be significantly ($P<0.05$) different from the control cells. Between 0.1 mg Cu/L and 1 mg Cu/L the difference in % DNA in the comet tails was not significantly different. Results of cells exposed to 50 mg Cu/L may be considered differently due to the cytotoxicity assays showing cells at this concentration to be (apoptotic/necrotic) nonviable meaning that the DNA damage was most probably the result of DNA breakdown due to cell death. Figure 4.15 shows tail % data in the form of histograms. Each dose is shown with the level of % DNA in the comet tail against the number of cells exhibiting that percentage.

Figure 4.16 shows a box plot of the data interpreted using the tail moment parameter. The data appears very similar to the % tail DNA. The ranges of the control and 1mg Cu/L dose appeared comparable whilst 0.1 and 10 mg Cu/L also showed comparable ranges to each other. 50 mg/L again showed a much higher range than all other parameters. Using this measure, all parameters were found to

be significantly different from the control and each other, except 0.1 and 1 mg Cu/L. Figure 4.17 shows the tail moment data in the form of histograms with each dose is shown with the level of tail moment against the number of cells exhibiting that moment.

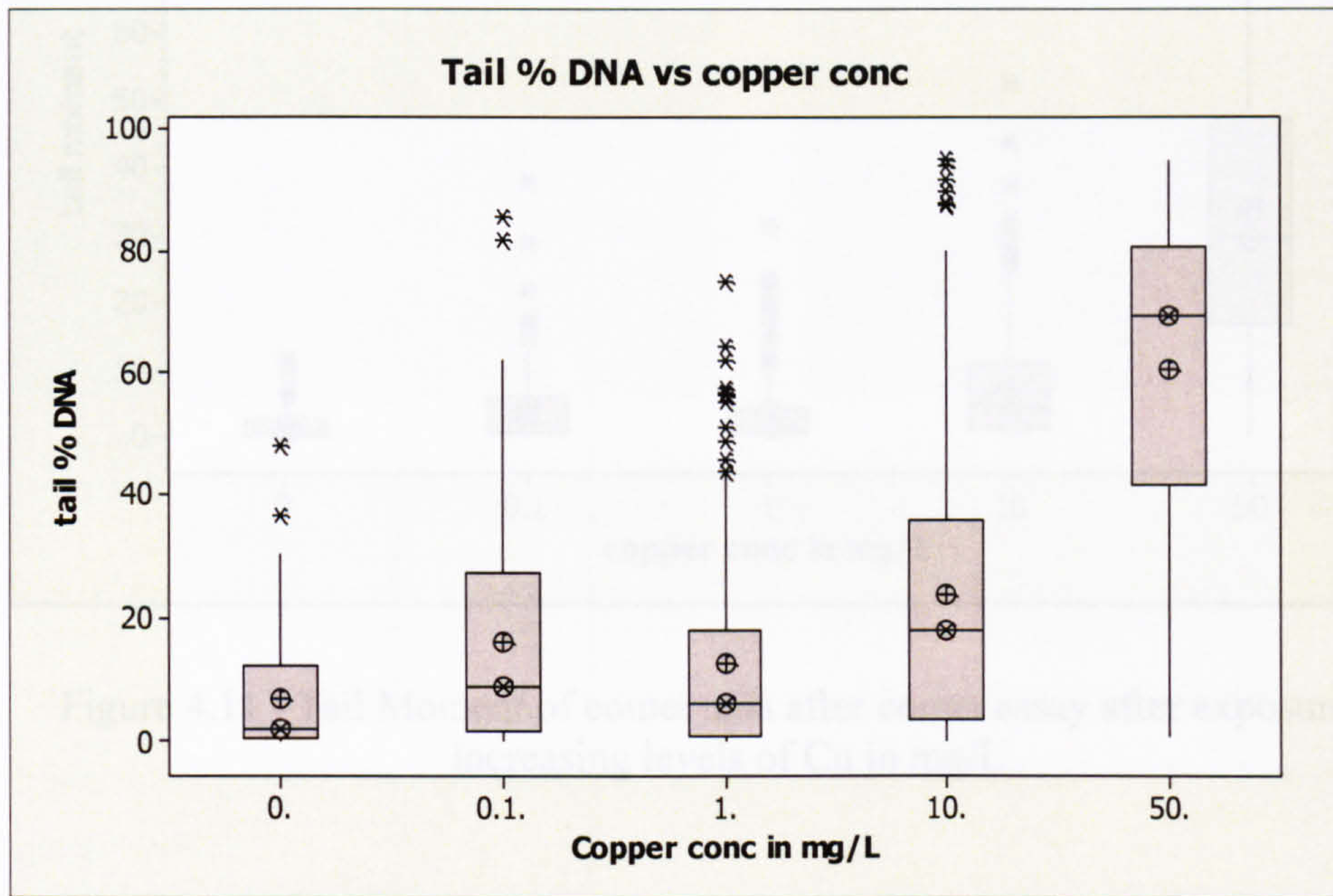


Figure 4.9 –Percentage of DNA found in comet tails after exposure to increasing levels of Cu (in mg/L)

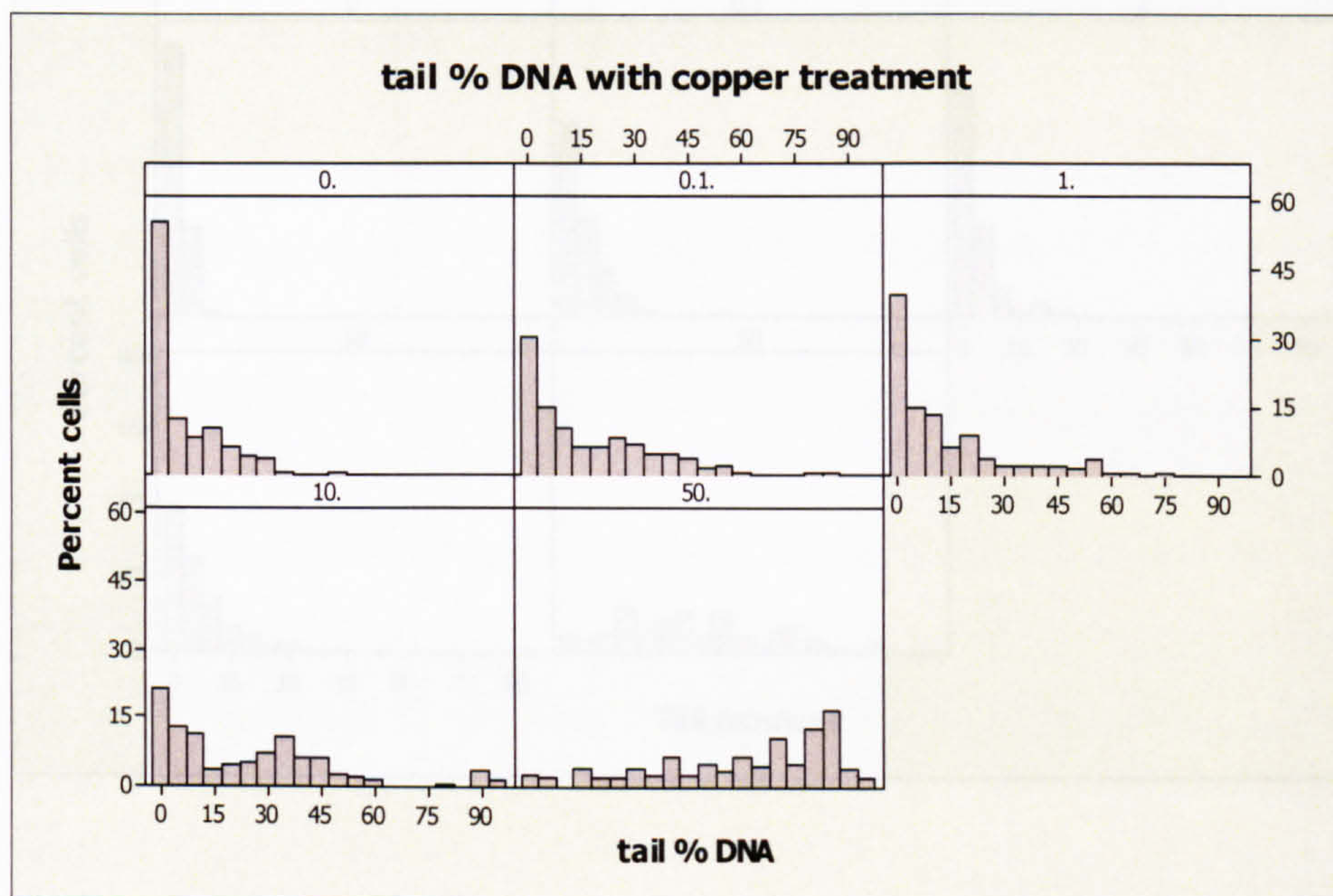


Figure 4.10 –Percentage of cells vs. percentage DNA in comet tails with increasing concentrations (in mg/L) of Cu. (n=3)

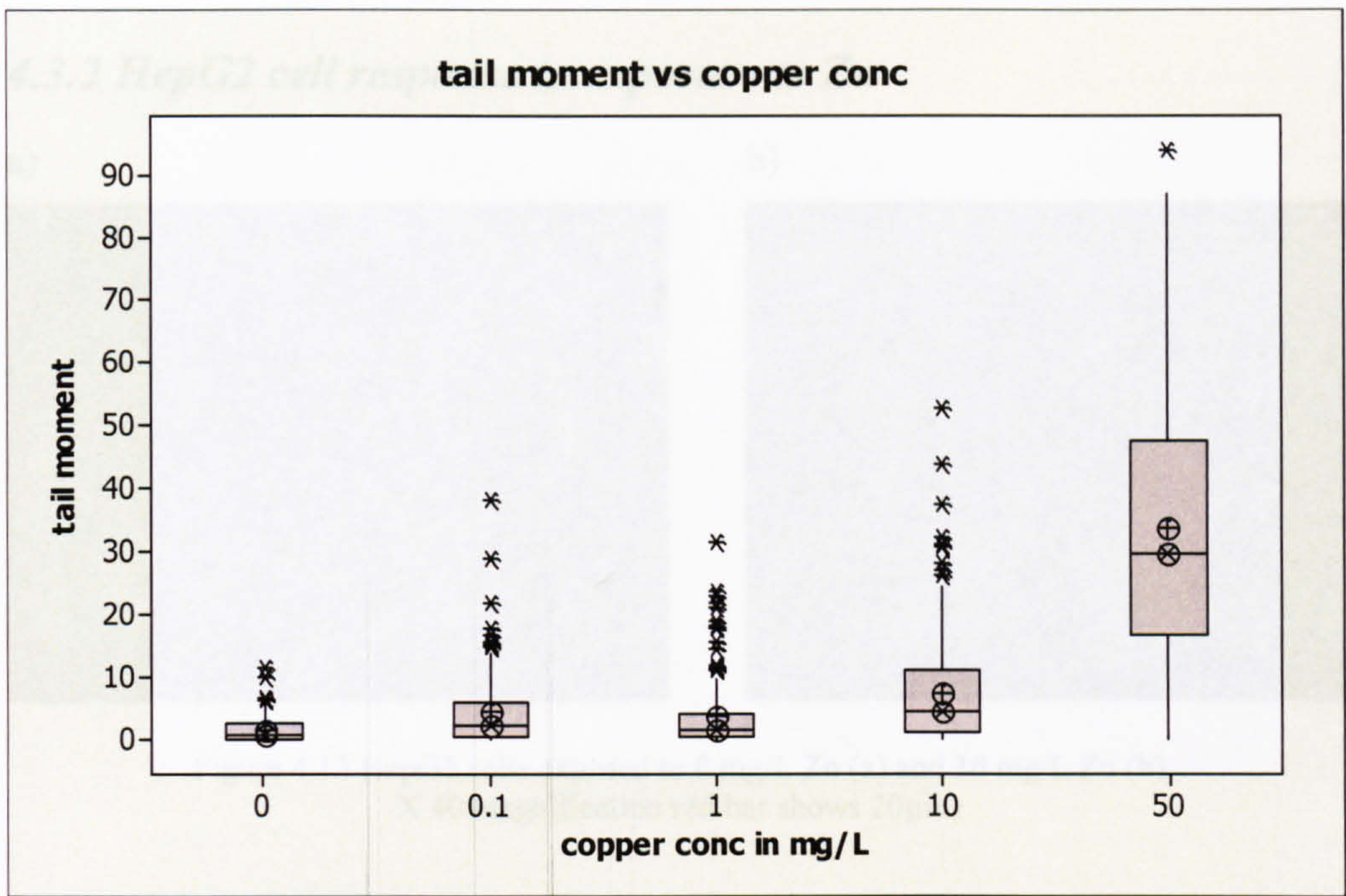


Figure 4.11 –Tail Moment of comet tails after comet assay after exposure to increasing levels of Cu in mg/L

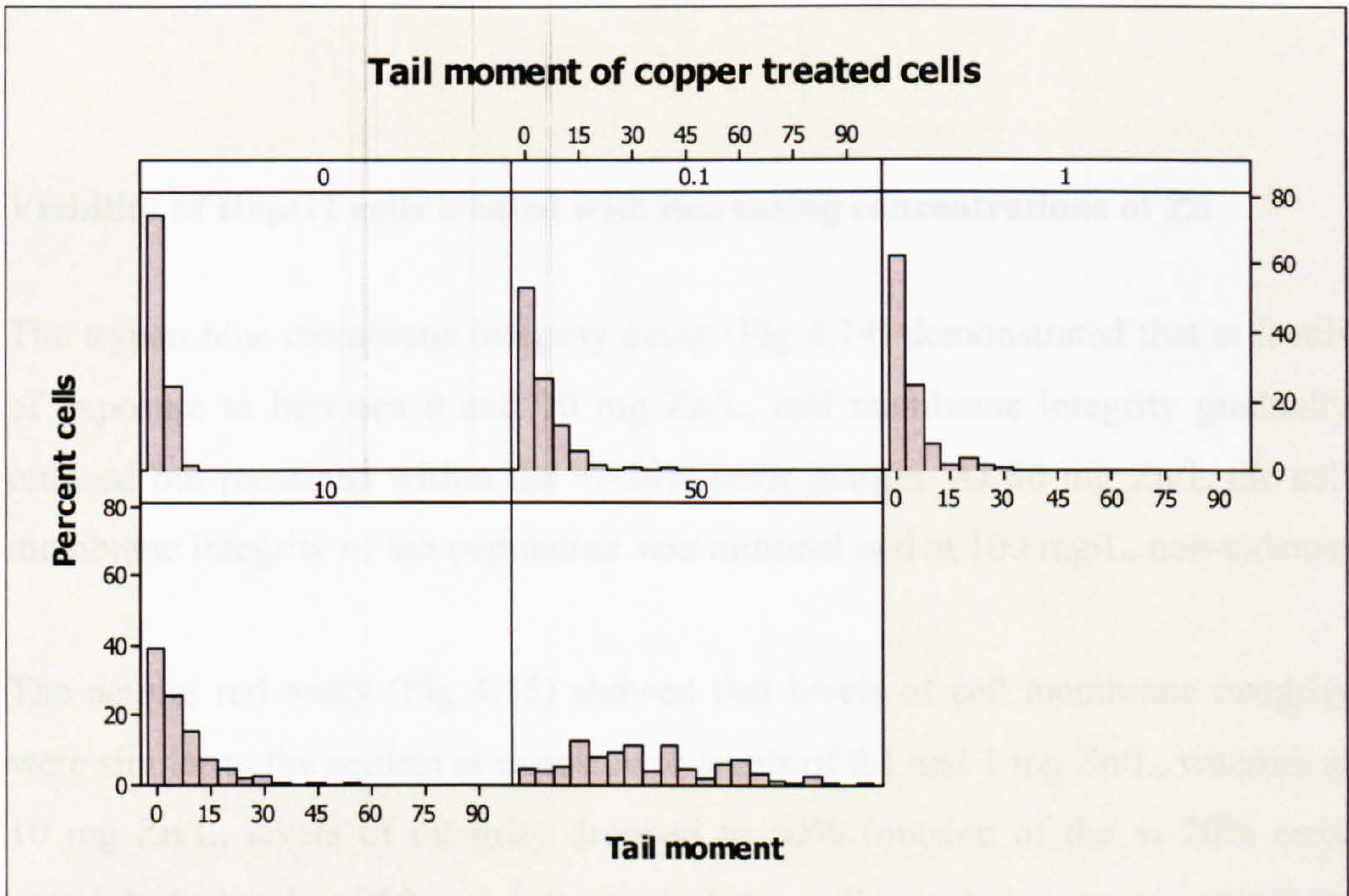


Figure 4.12 – Percentage of cells vs. Tail Moment of comet tails treatments with increasing concentrations (in mg/L) of Cu. (n=3)

4.3.2 HepG2 cell response to exposure to Zn

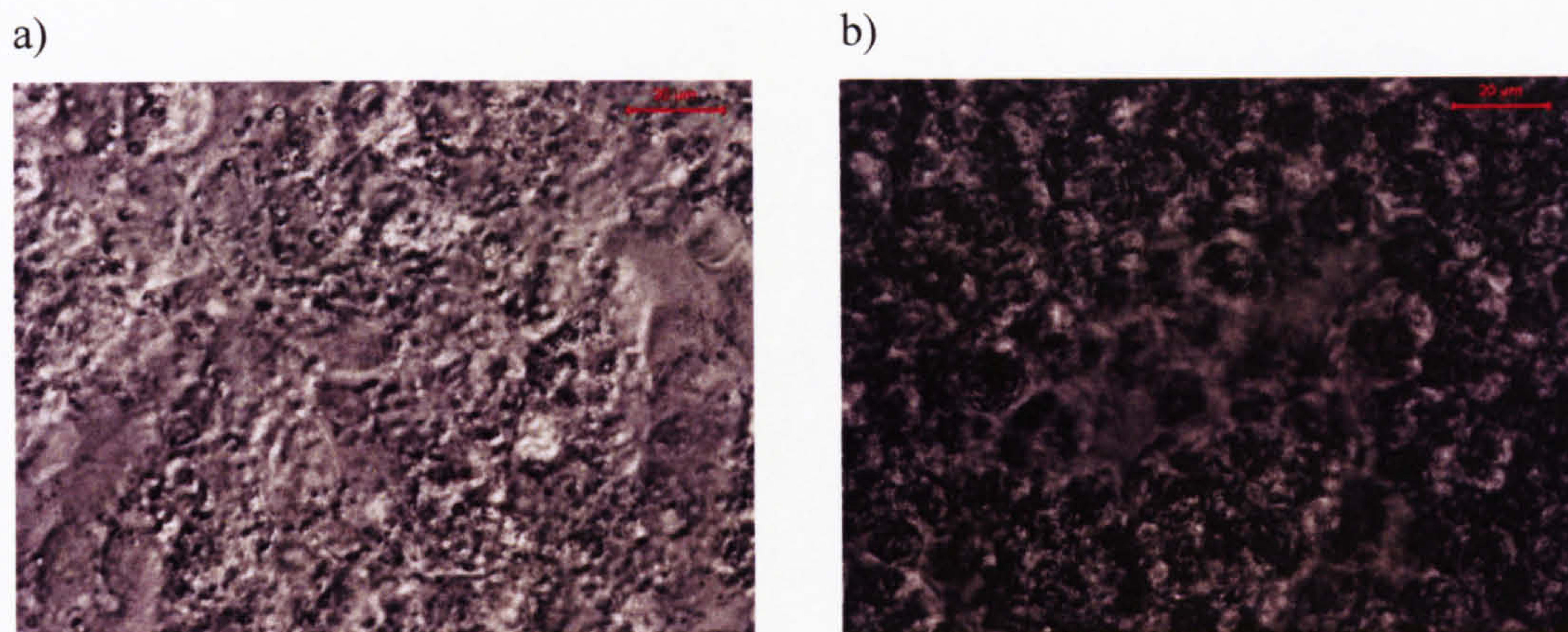


Figure 4.13 HepG2 cells exposed to 0 mg/L Zn (a) and 10 mg/L Zn (b)
X 40 magnification red bar shows 20μm)

Figure 4.13 shows images captured through a phase contrast microscope of control HepG2 cells that have not been exposed to Zn (a) and cells exposed to 10 mg/L Zn (b). The pictures illustrate the changes in cell morphology caused by the metal dose.

Viability of HepG2 cells treated with increasing concentrations of Zn

The trypan blue membrane integrity assay (Fig 4.14) demonstrated that at levels of exposure to between 0 and 10 mg Zn/L, cell membrane integrity gradually reduced but remained within the +/-20% error margin. At 50 mg Zn/L the cell membrane integrity of the population was minimal and at 100 mg/L, non-existent.

The neutral red assay (Fig 4.15) showed that levels of cell membrane integrity were similar to the control at exposure to levels of 0.1 and 1 mg Zn/L, whereas at 10 mg Zn/L, levels of integrity dropped to 50% (outside of the +/-20% error margin). At levels of 50 and 100 mg Zn/L the cell populations were essentially dead.

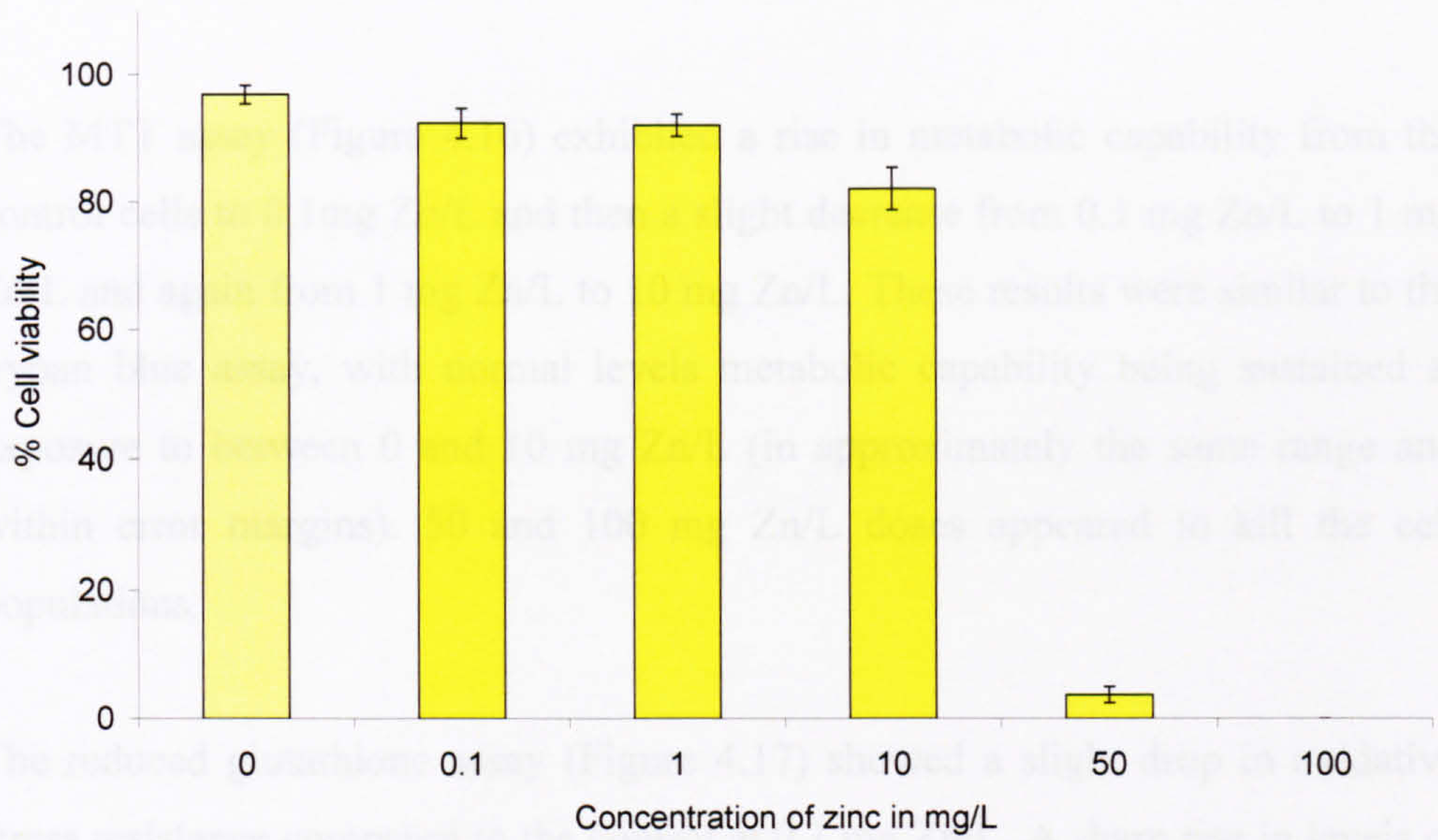


Figure 4.14 HepG2 cells exposed to 0-100 mg/L of Zn for 24hrs;
live/dead assessment using the Trypan blue dye exclusion cell viability assay.
Results are expressed as a percentage of the control +/-SEM (n=3)

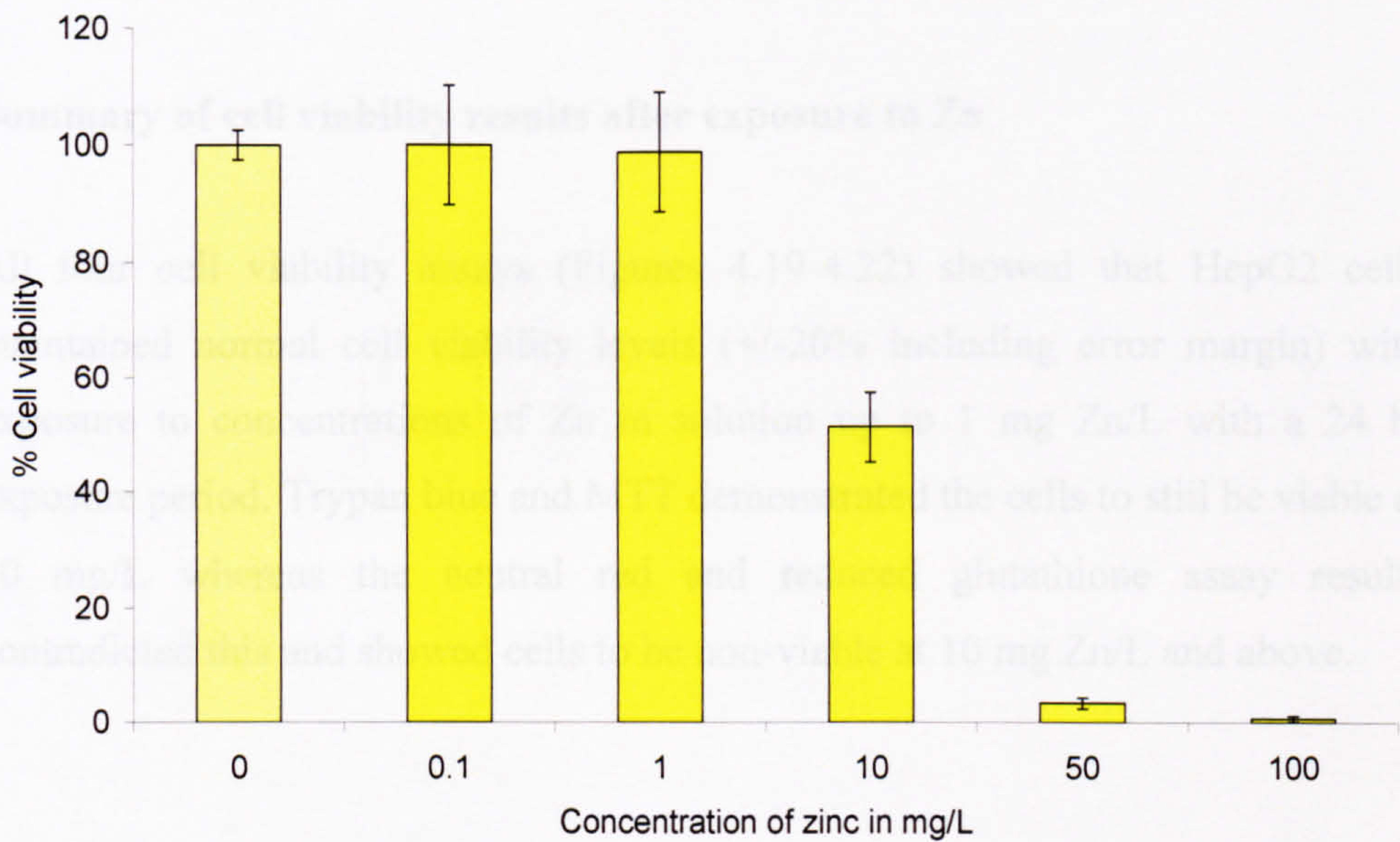


Figure 4.15 HepG2 cells exposed to 0-100 mg/L of Zn for 24hrs;
live/dead assessment using the Neutral Red dye uptake cell viability assay.
Results are expressed as a percentage of the control +/-SEM (n=3)

The MTT assay (Figure 4.16) exhibited a rise in metabolic capability from the control cells to 0.1mg Zn/L and then a slight decrease from 0.1 mg Zn/L to 1 mg Zn/L and again from 1 mg Zn/L to 10 mg Zn/L. These results were similar to the trypan blue assay, with normal levels metabolic capability being sustained at exposure to between 0 and 10 mg Zn/L (in approximately the same range and within error margins). 50 and 100 mg Zn/L doses appeared to kill the cell populations.

The reduced glutathione assay (Figure 4.17) showed a slight drop in oxidative stress resistance compared to the control at 0.1 mg Zn/L. A sharp rise in levels of reduced glutathione/mg protein was seen at exposure to 1 mg Zn/L whereas at 10 mg/L and above, no glutathione remained i.e. no cell viability. The viability range was similar to that shown by the neutral red assay.

Summary of cell viability results after exposure to Zn

All four cell viability assays (Figures 4.19-4.22) showed that HepG2 cells maintained normal cell viability levels (+/-20% including error margin) with exposure to concentrations of Zn in solution up to 1 mg Zn/L with a 24 hr exposure period. Trypan blue and MTT demonstrated the cells to still be viable at 10 mg/L whereas the neutral red and reduced glutathione assay results contradicted this and showed cells to be non-viable at 10 mg Zn/L and above.

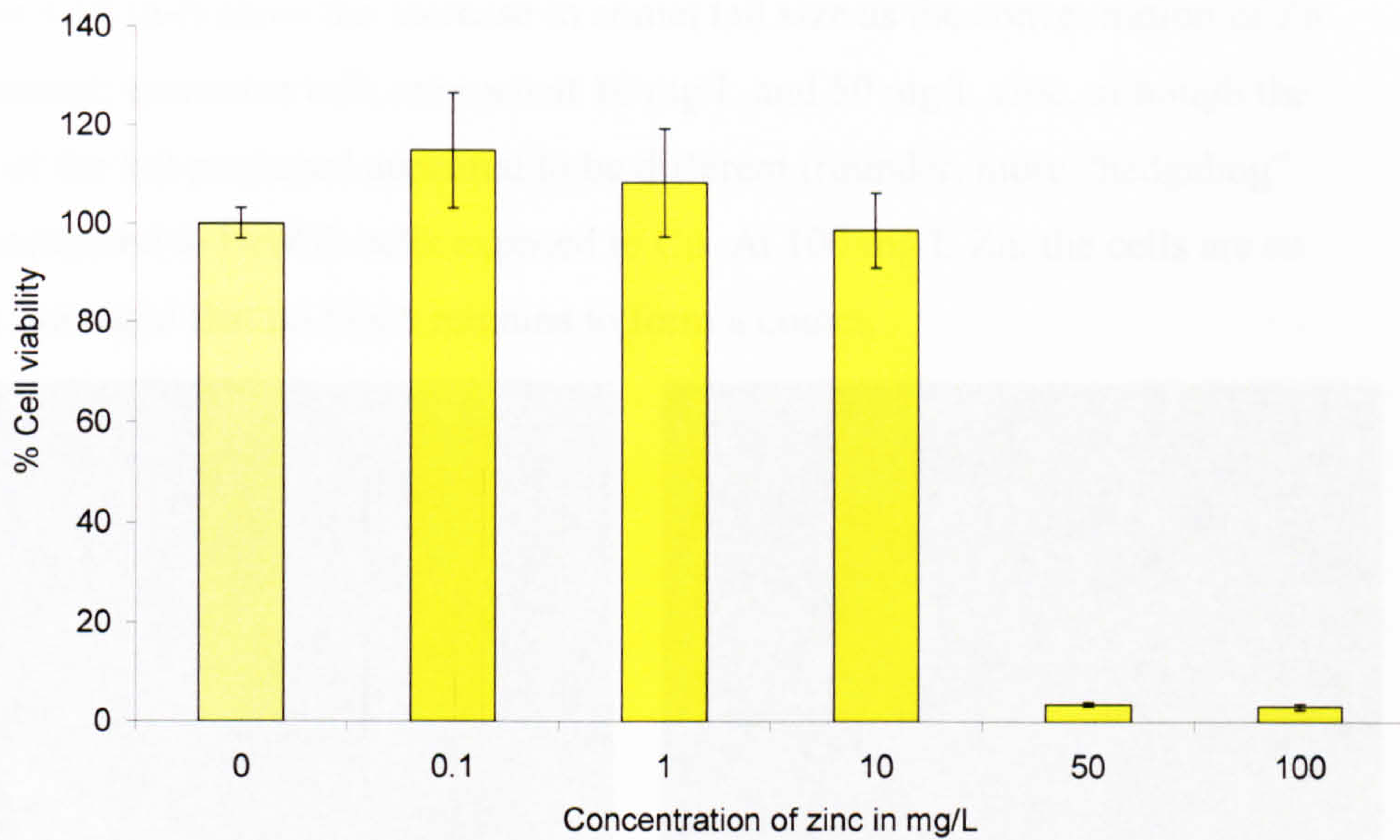


Figure 4.16 HepG2 cells exposed to 0-100 mg/L of Zn for 24 hrs;
live/dead assessment using the MTT cell viability assay.
Results are expressed as a percentage of the control +/-SEM (n=3)

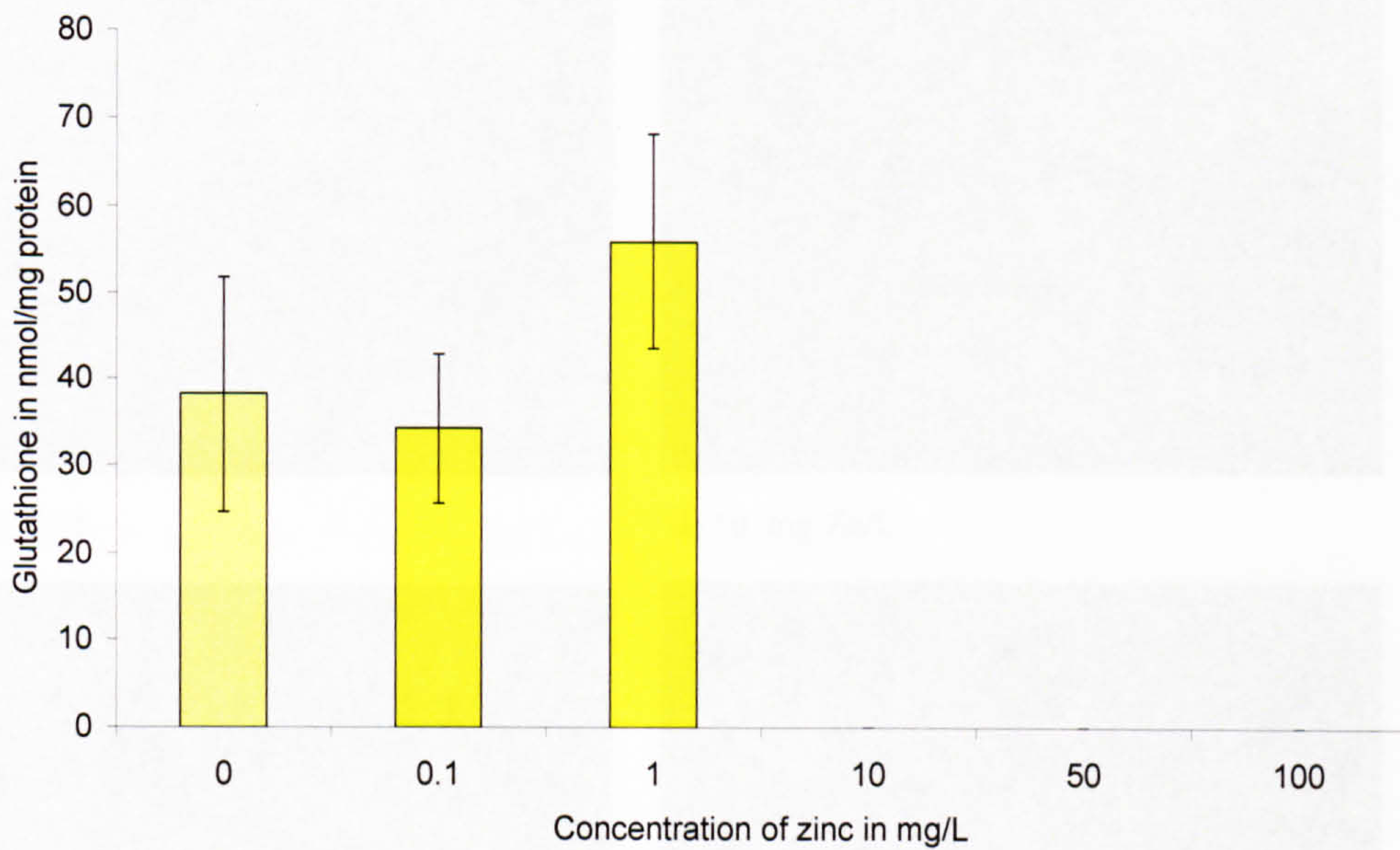
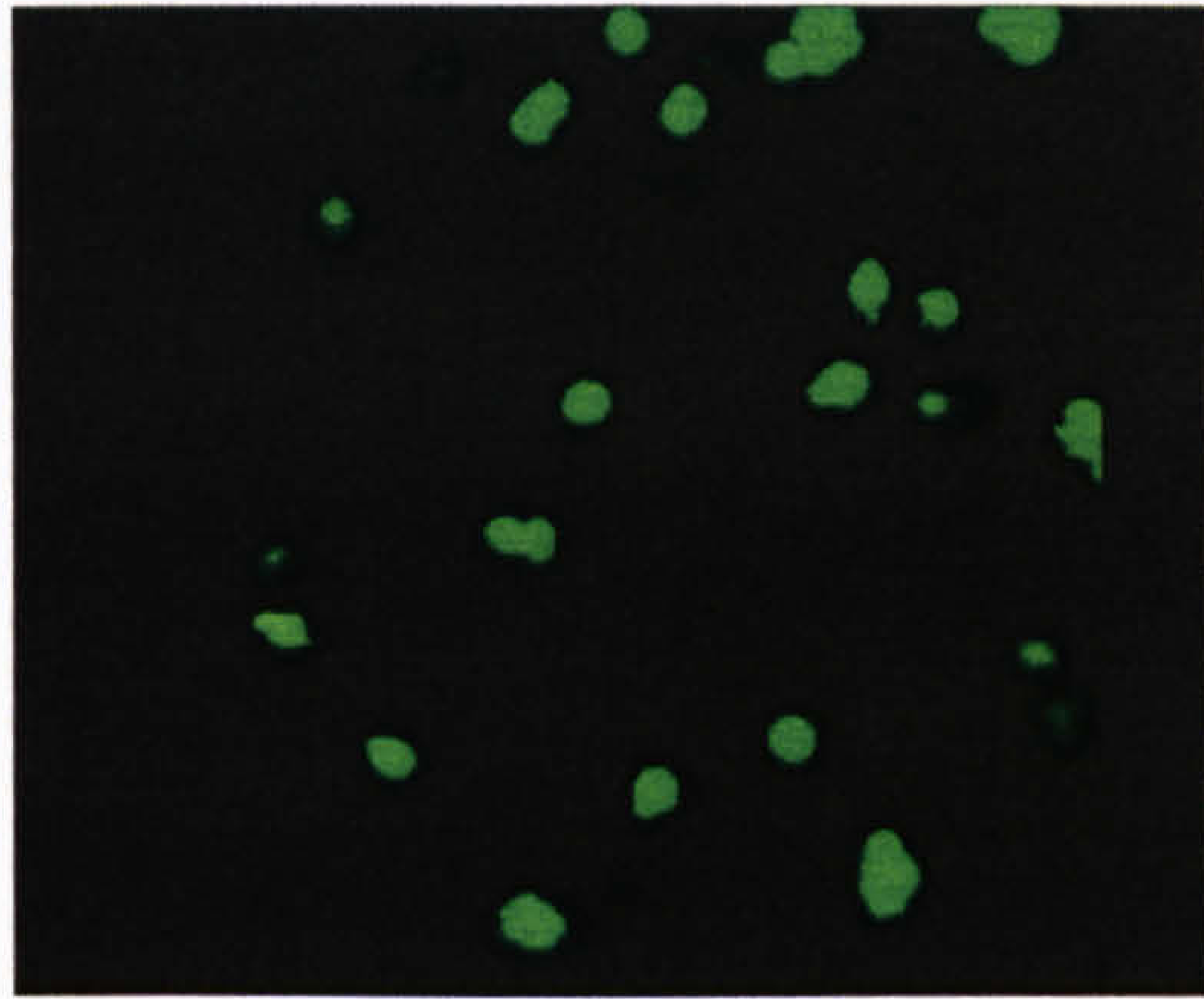


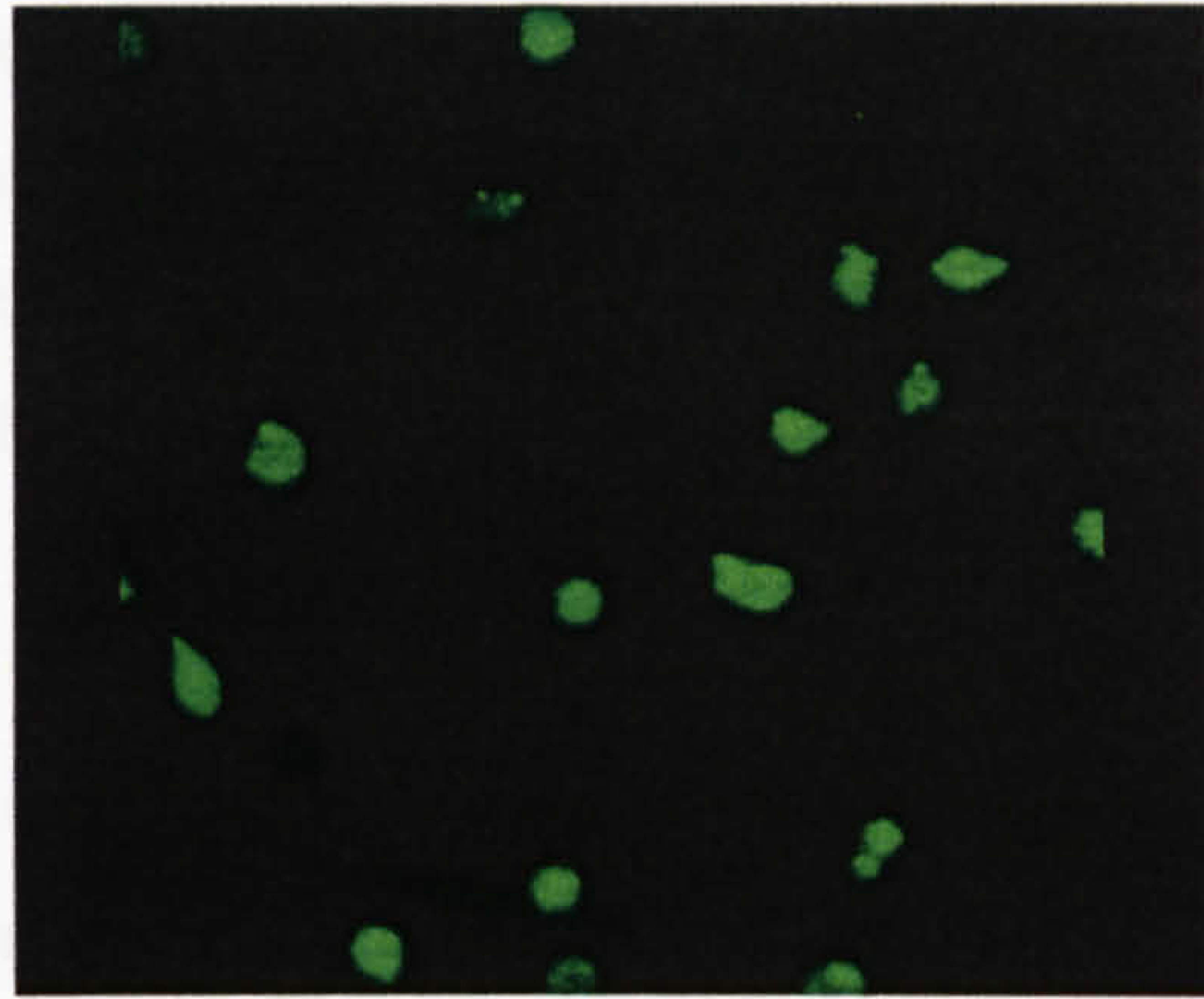
Figure 4.17 HepG2 cells exposed to 0-100 mg/L of Zn for 24 hrs;
reduced glutathione assay.
Results are expressed in nmol GSH/mg protein +/-SEM (n=3)

Comet images of HepG2 cells exposed to increasing concentrations of Zn

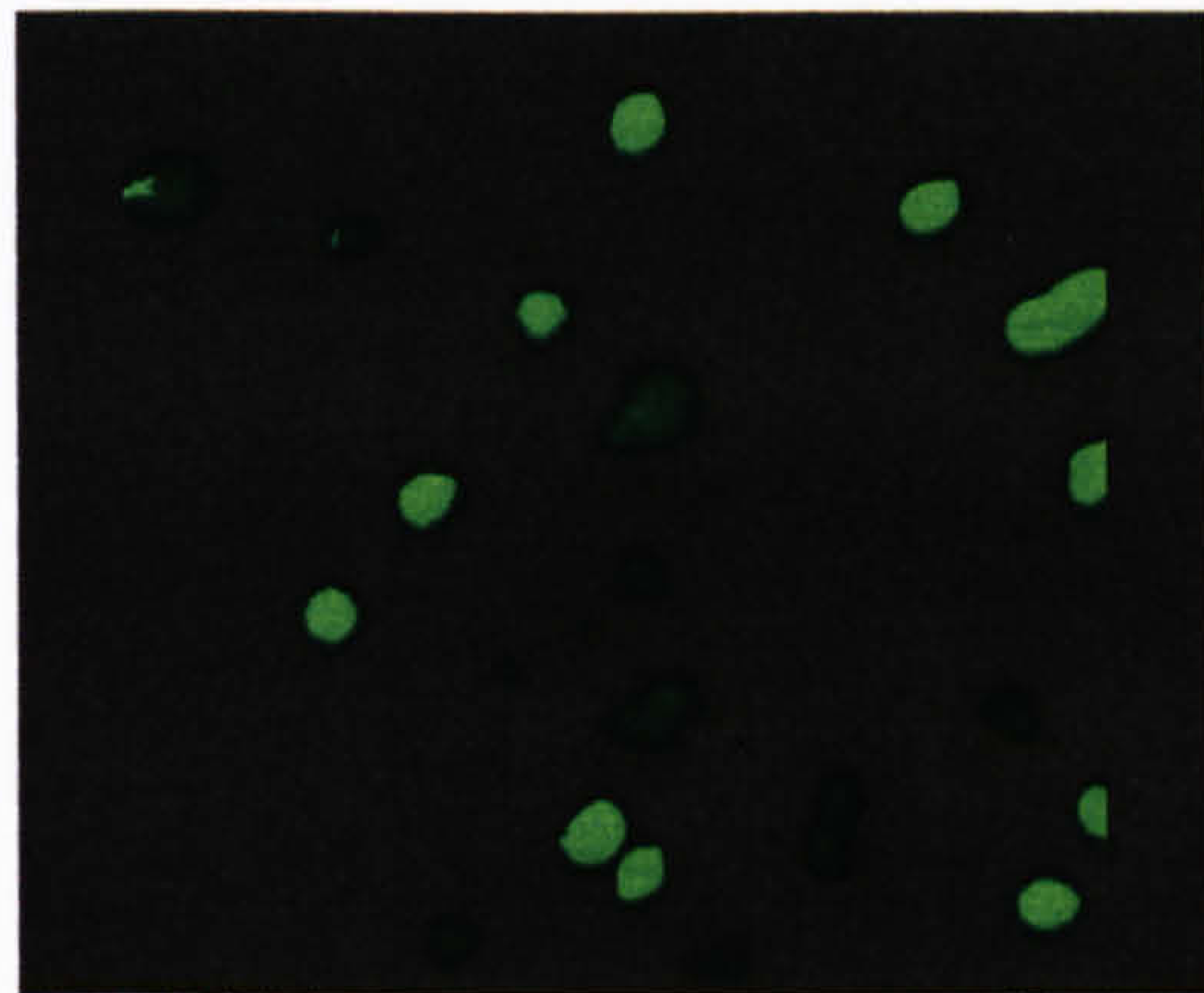
Figures 4.18 (a-f) show the increase in comet tail size as the concentration of Zn is increased; extensive tails are seen at 10 mg/L and 50 mg/L zinc, although the shape of the tail produced appeared to be different (rounder, more "hedgehog" like) compared to HepG2 cells exposed to Cu. At 100 mg/L Zn, the cells are so highly damaged that no DNA remains to form a comet.



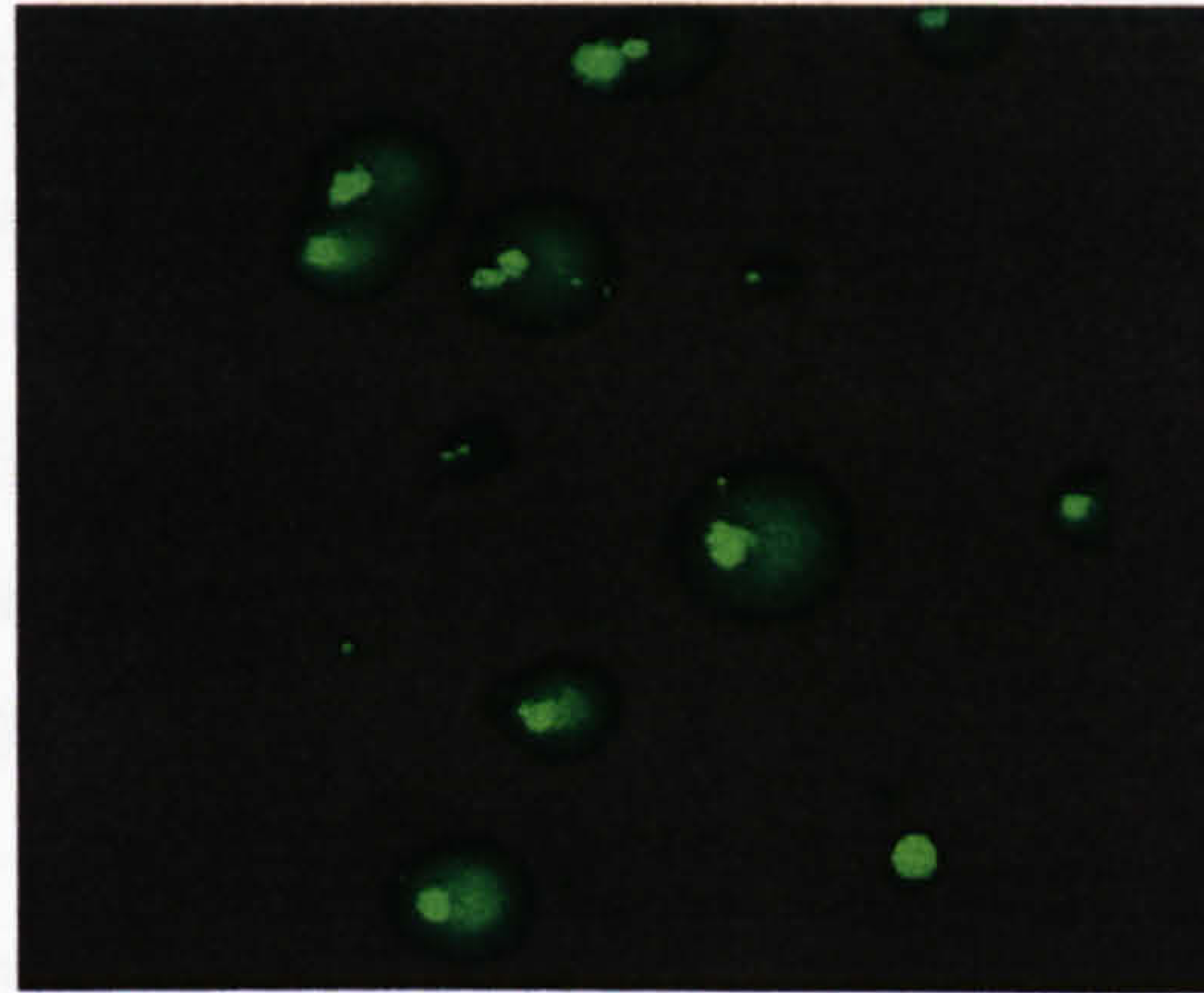
a) 0 mg Zn/L



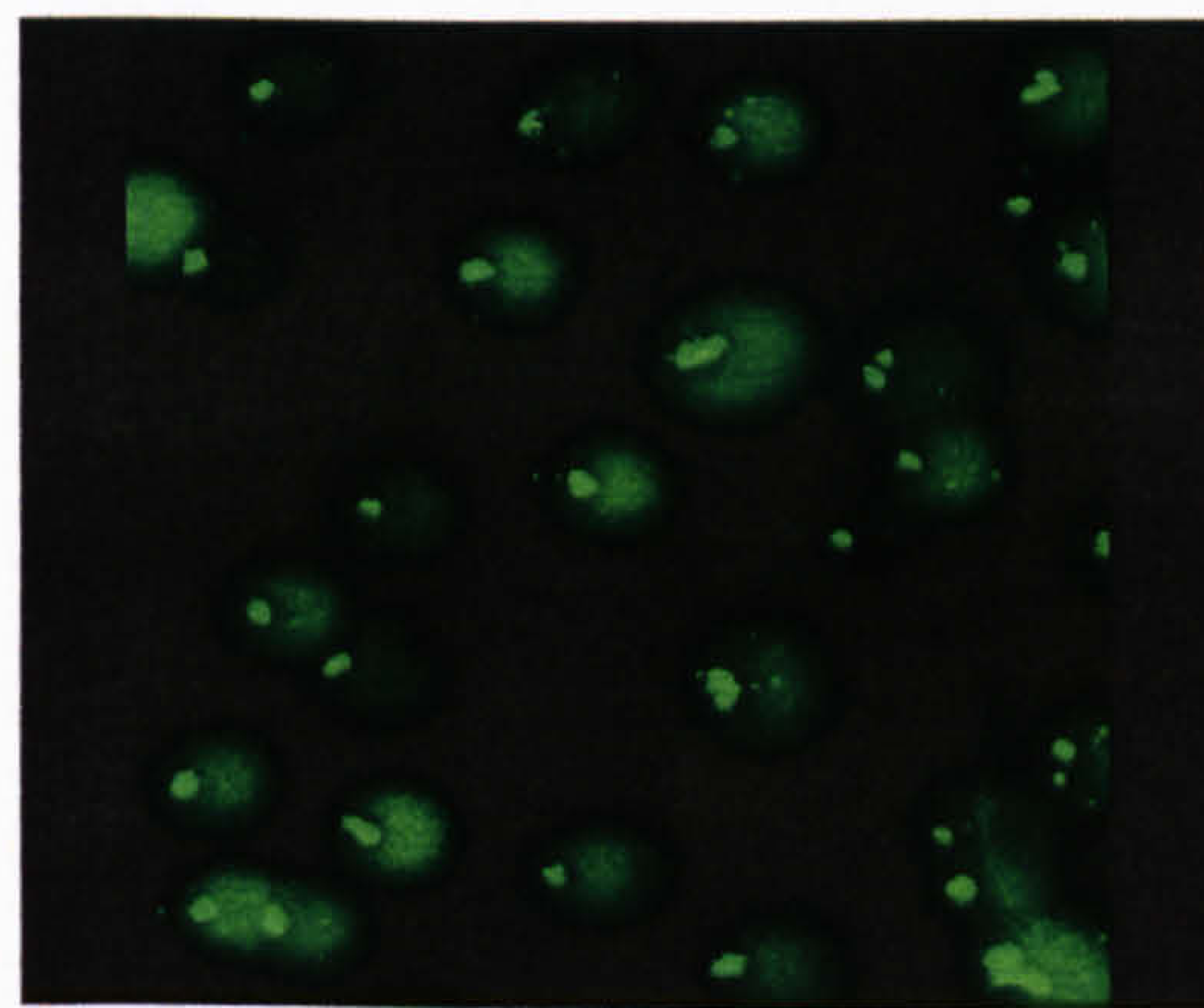
b) 0.1 mg Zn/L



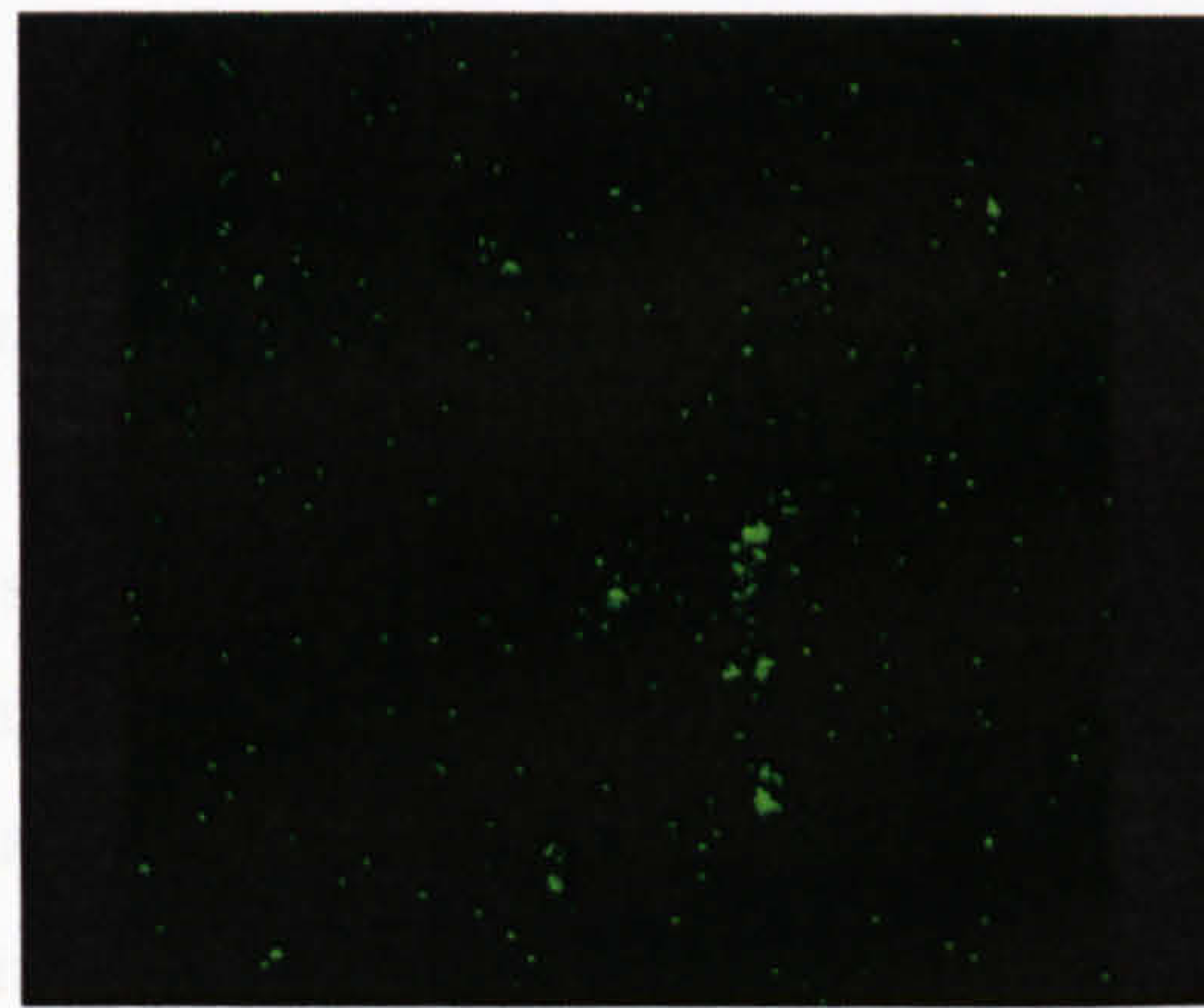
c) 1 mg Zn/L



d) 10 mg Zn/L



e) 50 mg Zn/L



f) 100 mg Zn/L

Figure 4.18 Confocal microscope images of HepG2 cells exposed to increasing concentrations of Zn. Direction of electrophoresis was left to right

DNA damage to HepG2 cells treated with increasing levels of Zn

Figures 4.19 to 4.22 show the effects of increasing Zn concentration on HepG2 cells, having been put through the comet assay, again examining two parameters; % tail DNA and tail moment. Results of cells exposed to 10 mg/L and 50 mg/L need to be considered differently due to the cytotoxicity assays showing cells at this concentration may be (at 10mg/L) or definitely are (at 50 mg/L) (apoptotic/necrotic) nonviable.

Figure 4.19 shows the box plot of tail % as Zn concentration increases. Normal levels of % DNA in the comet tails for the control cells were 0-15%. The main range of % DNA in the comet tails increased when exposed to 0.1 mg Zn/L to 5-30%. This level was significantly ($P<0.05$) different from the control. The range of % DNA in the comet tails when exposed to 1 mg Zn/L (5-25%) decreased slightly compared to 0.1 mg Zn/L but the difference in levels was between the 2 concentrations was not found to be significantly different. Between 1 mg/L and 10 mg/L the levels of DNA damage increased greatly (55-85% range). All conditions were found to be significantly different from the control and each other (except 0.1 mg Zn/L and 1 mg Zn/L). Figure 4.20 shows tail % data in the form of histograms. Each dose is shown with the level of % DNA in the comet tail against the number of cells exhibiting that percentage.

Figure 4.21 shows the box plot of tail moment as Zn concentration increases. The findings look very similar to those results generated using the tail % measure; in this case all conditions were found to be significantly different from the control and from each other including 0.1 mg/L and 1 mg/L. Figure 4.22 shows the tail moment data in the form of histograms. Each dose is shown with the level of tail moment against the number of cells exhibiting that moment.

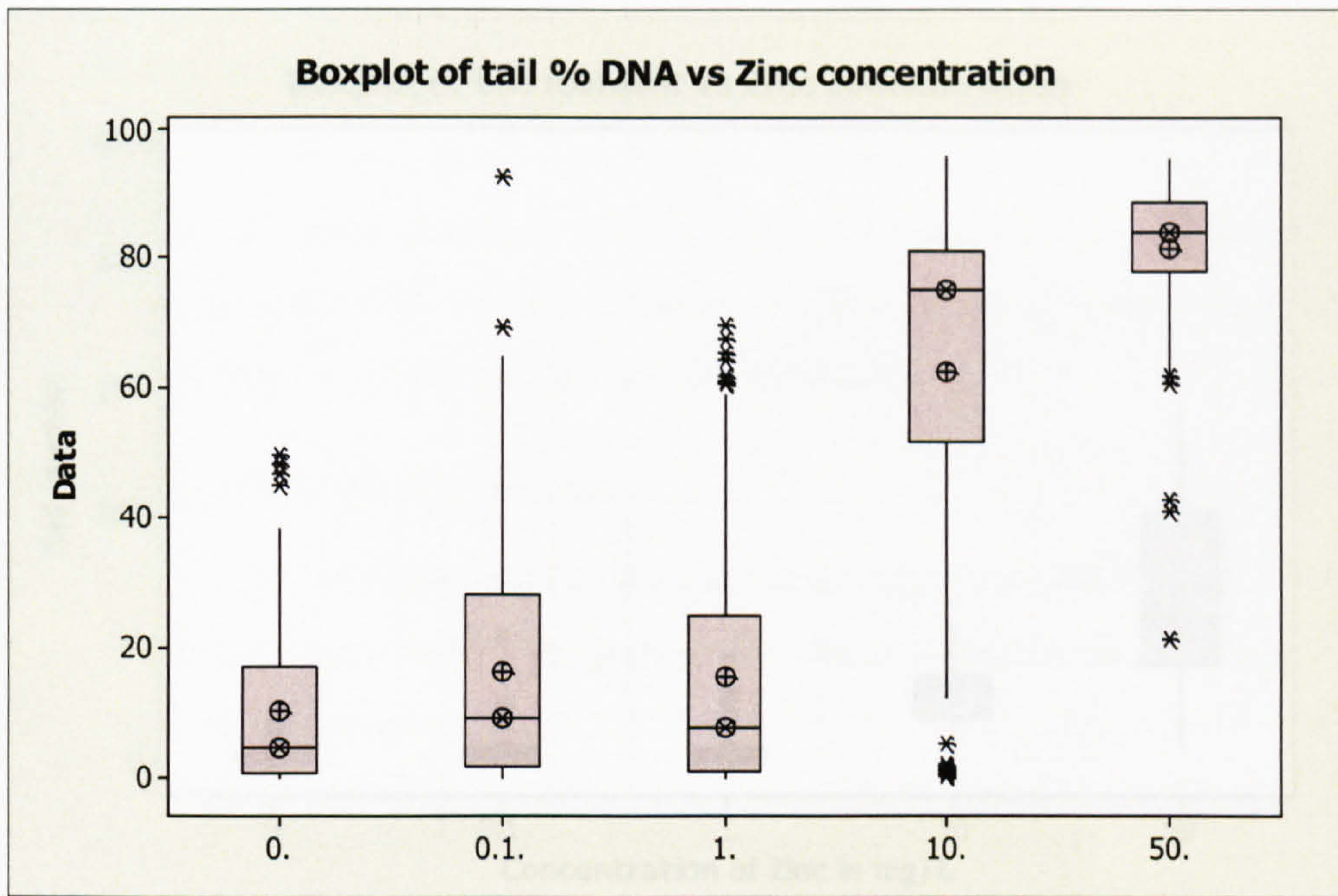


Figure 4.19 Percentage of DNA found in comet tails after exposure to increasing levels of Zn (in mg/L)

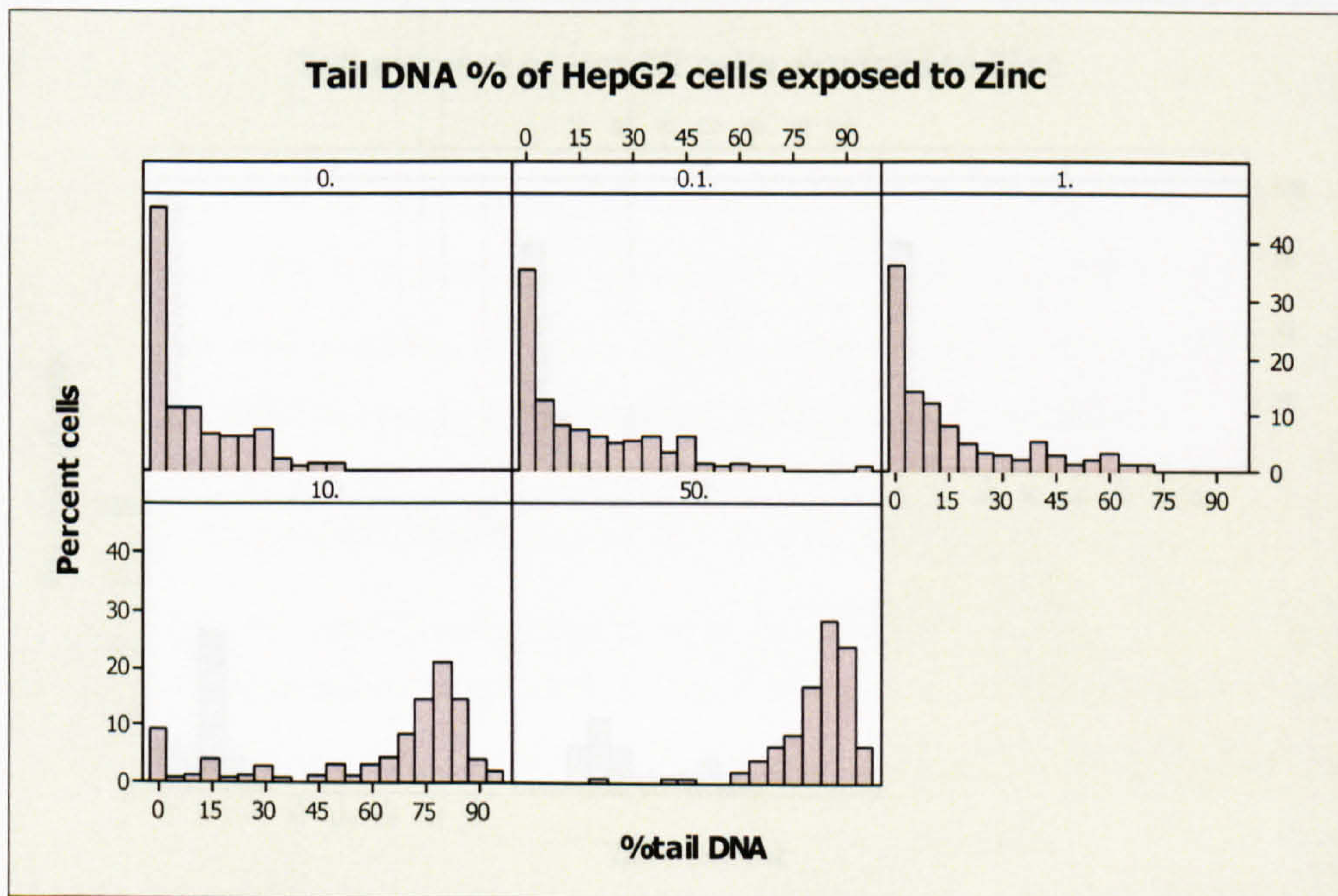


Figure 4.20 –Percentage of cells vs. percentage DNA in comet tails with increasing concentrations (in mg/L) of Zn. (n=3)

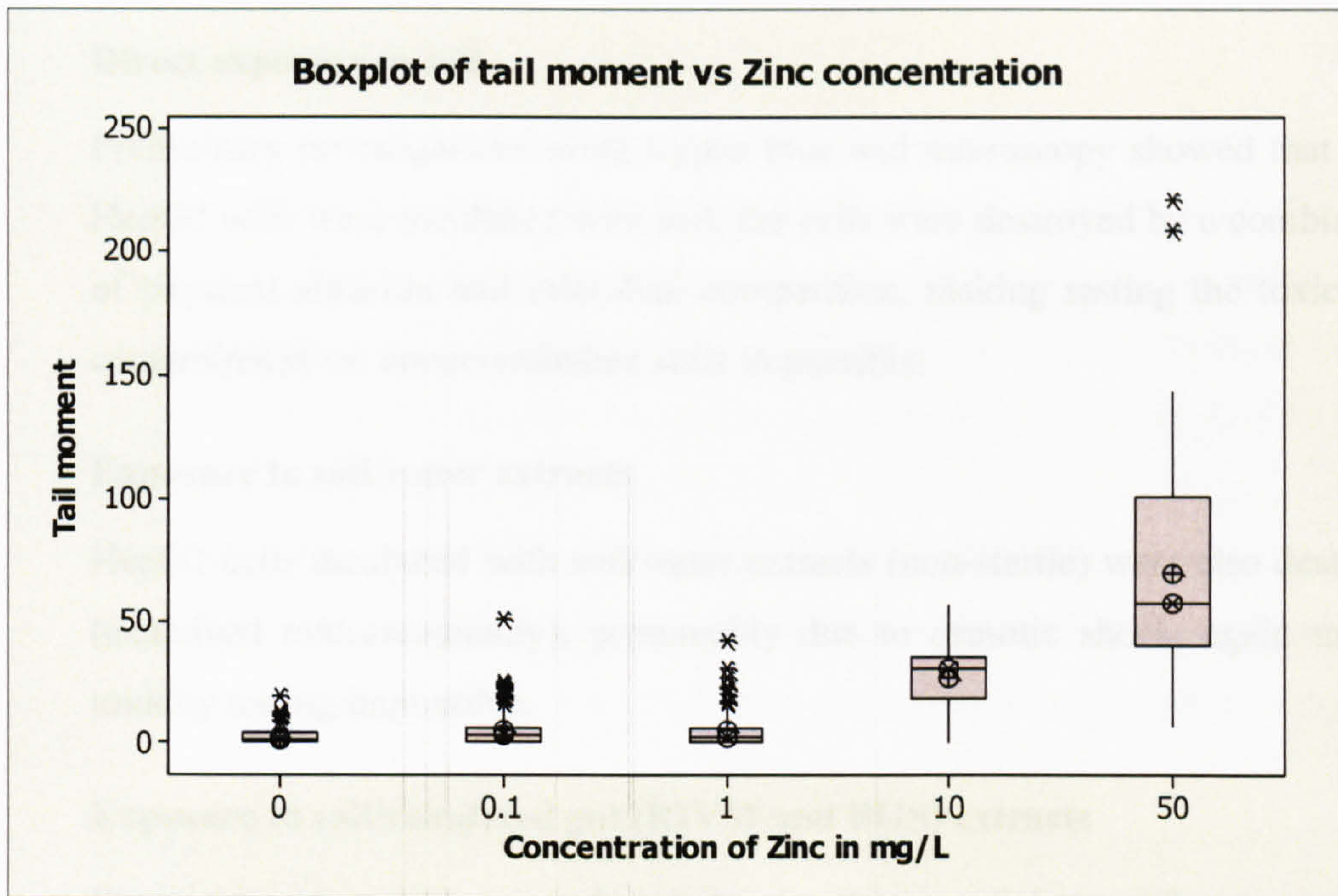


Figure 4.21 Tail Moment of comet tails after comet assay after exposure to increasing levels of Zn in mg/L

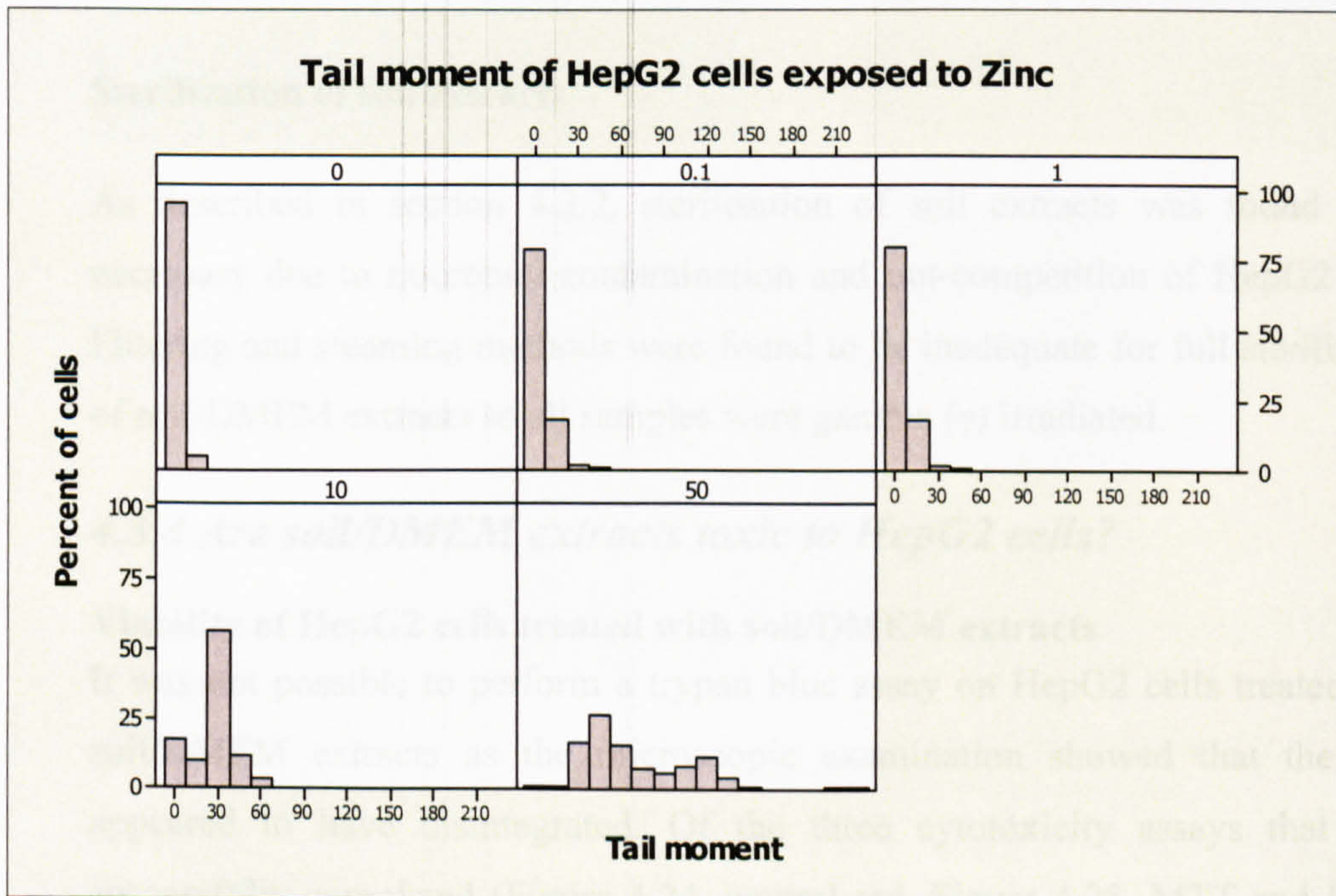


Figure 4.22 Percentage of cells vs. Tail Moment of comet tails treatments with increasing concentrations (in mg/L) of Zn. (n=3)

4.3.3 Soil and soil extracts exposure to HepG2 cells

Direct exposure to soil

Preliminary investigations using trypan blue and microscopy showed that when HepG2 cells were incubated with soil, the cells were destroyed by a combination of physical abrasion and microbial competition, making testing the toxicity of contaminated vs. uncontaminated soils impossible.

Exposure to soil/water extracts

HepG2 cells incubated with soil/water extracts (non-sterile) were also destroyed (examined microscopically); presumably due to osmotic shock, again making toxicity testing impossible.

Exposure to soil/simulated gut (RIVM and BGS) extracts

Examination by microscopy found that incubation of these soil extracts (non-sterile) with human cells was also unsuccessful. HepG2 cells were entirely broken down (digested?) by either unsuitable pH ("stomach" phase of the BGS extract) or the organic components (digestive enzymes?) of the extract solutions.

Sterilisation of soil extracts

As described in section 4.2.2, sterilisation of soil extracts was found to be necessary due to microbial contamination and out-competition of HepG2 cells. Filtering and steaming methods were found to be inadequate for full sterilisation of soil/DMEM extracts so all samples were gamma (γ) irradiated.

4.3.4 Are soil/DMEM extracts toxic to HepG2 cells?

Viability of HepG2 cells treated with soil/DMEM extracts

It was not possible to perform a trypan blue assay on HepG2 cells treated with soil/DMEM extracts as the microscopic examination showed that the cells appeared to have disintegrated. Of the three cytotoxicity assays that were successfully completed (Figure 4.24, neutral red, Figure 4.25, MTT and Figure 4.26, reduced glutathione) two consistent themes emerged. Firstly, that HepG2 cells in irradiated DMEM (no soil), appeared to have similar viability to "control" HepG2 cells in non-irradiated DMEM. Secondly that soil/irradiated

DMEM solutions were generally cytotoxic to HepG2 cells, regardless of levels of metal contamination known to be present in the soils.

Cells treated with soil/DMEM extract (regardless of any level of metal contamination) exhibited different levels of viability dependant on the assay examined. The results generated by the cytotoxicity assays that showed cell populations in irradiated DMEM were similar to those in non-irradiated DMEM were contradicted by both the phase contrast (Figure 4.23) and confocal microscope pictures (Figure 4.27) which showed obvious changes in cell morphology and high levels of cell debris in solution. Despite this the comet assay was performed but analysis was not possible due to limitations in the analysis software (debris surrounding DNA does not allow for measurement).

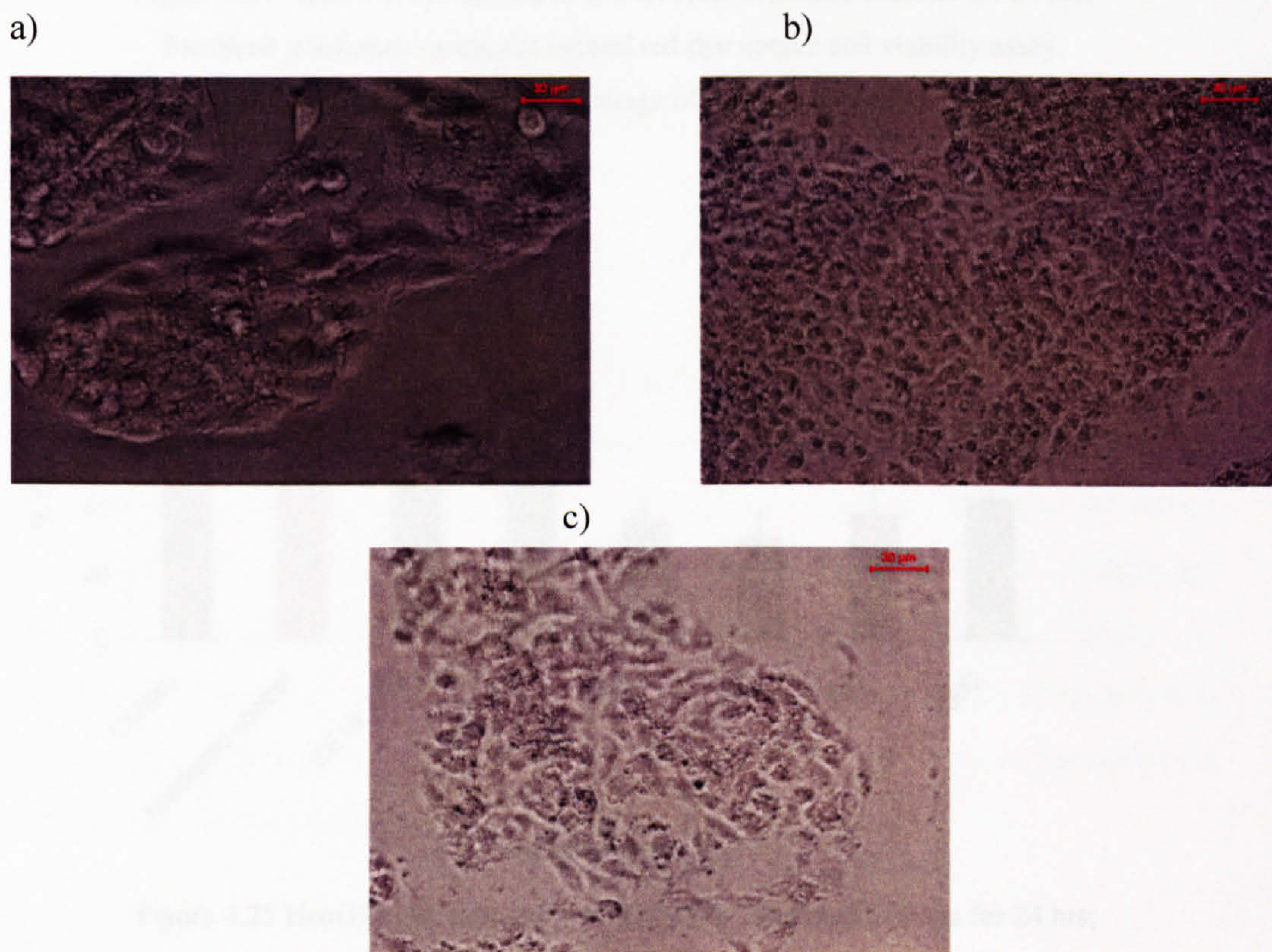


Figure 4.23 Phase contrast images of HepG2 cells exposed to non-irradiated DMEM (a), irradiated DMEM (b) and NE26,500/DMEMsolution - irradiated (c)

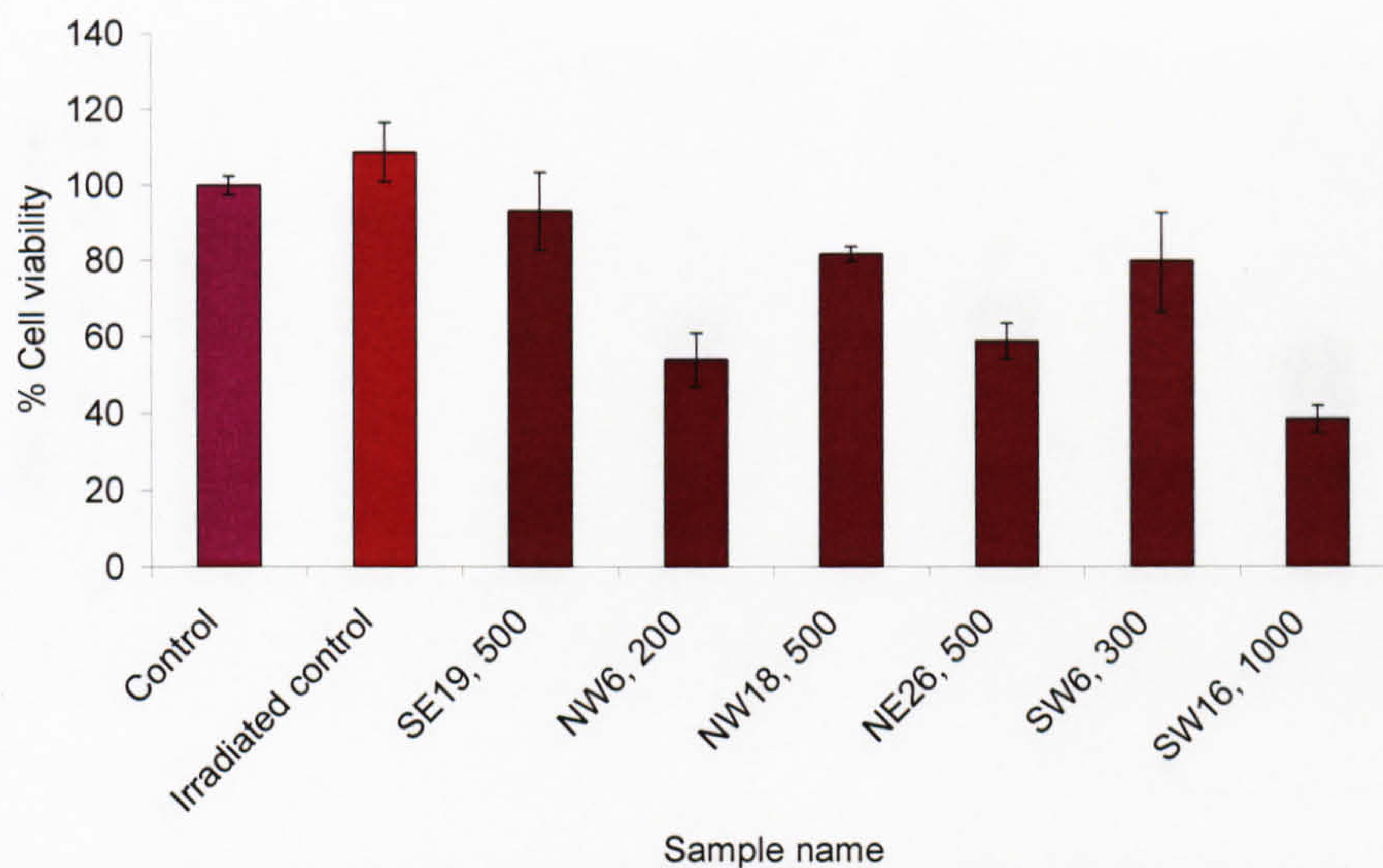


Figure 4.24 HepG2 cells exposed to soil/DMEM irradiated extracts for 24 hrs; live/dead assessment using the neutral red dye uptake cell viability assay. Results are expressed as a percentage of the control +/-SEM (n=3)

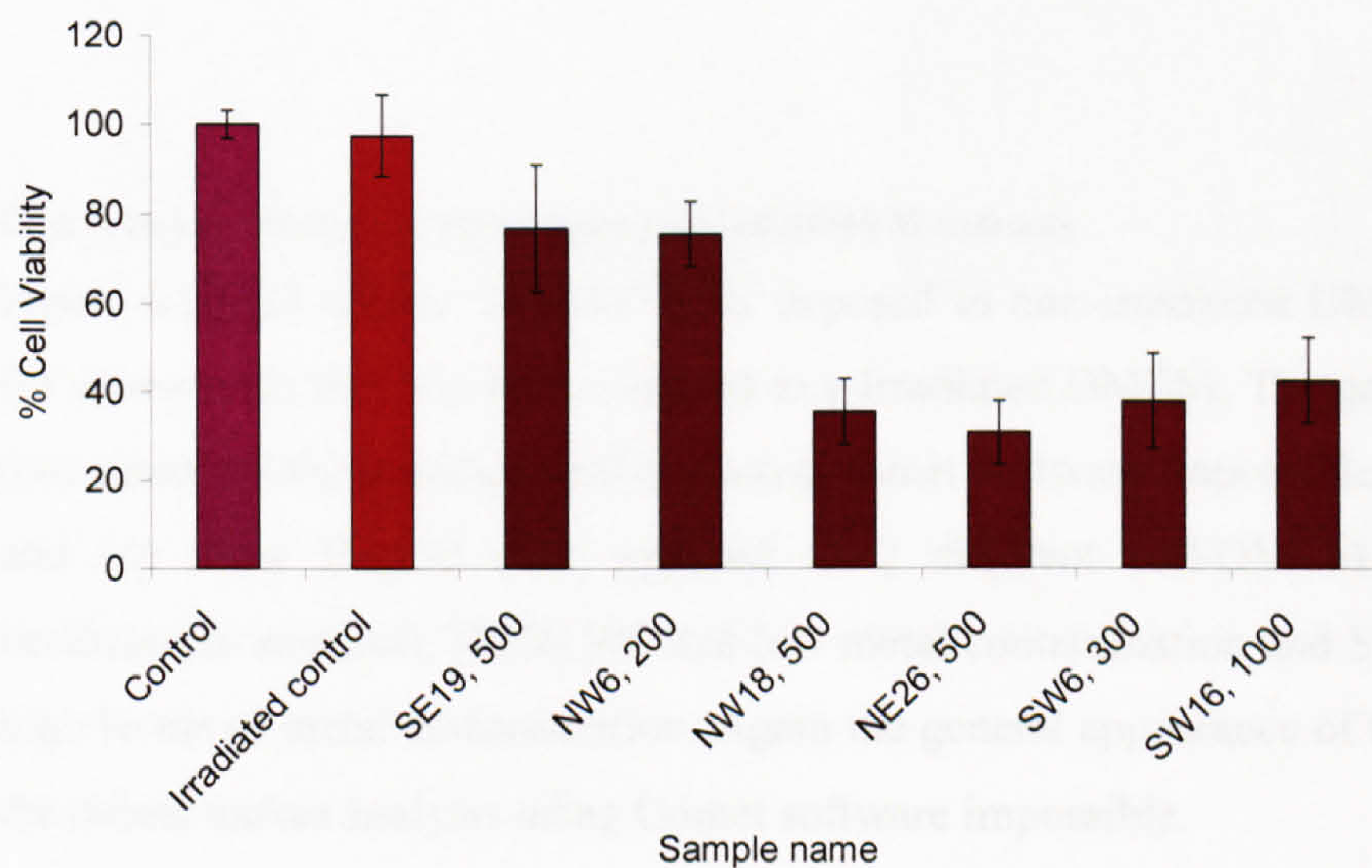


Figure 4.25 HepG2 cells exposed to soil/DMEM irradiated extracts for 24 hrs; live/dead assessment using the MTT cell viability assay. Results are expressed as a percentage of the control +/-SEM (n=3)

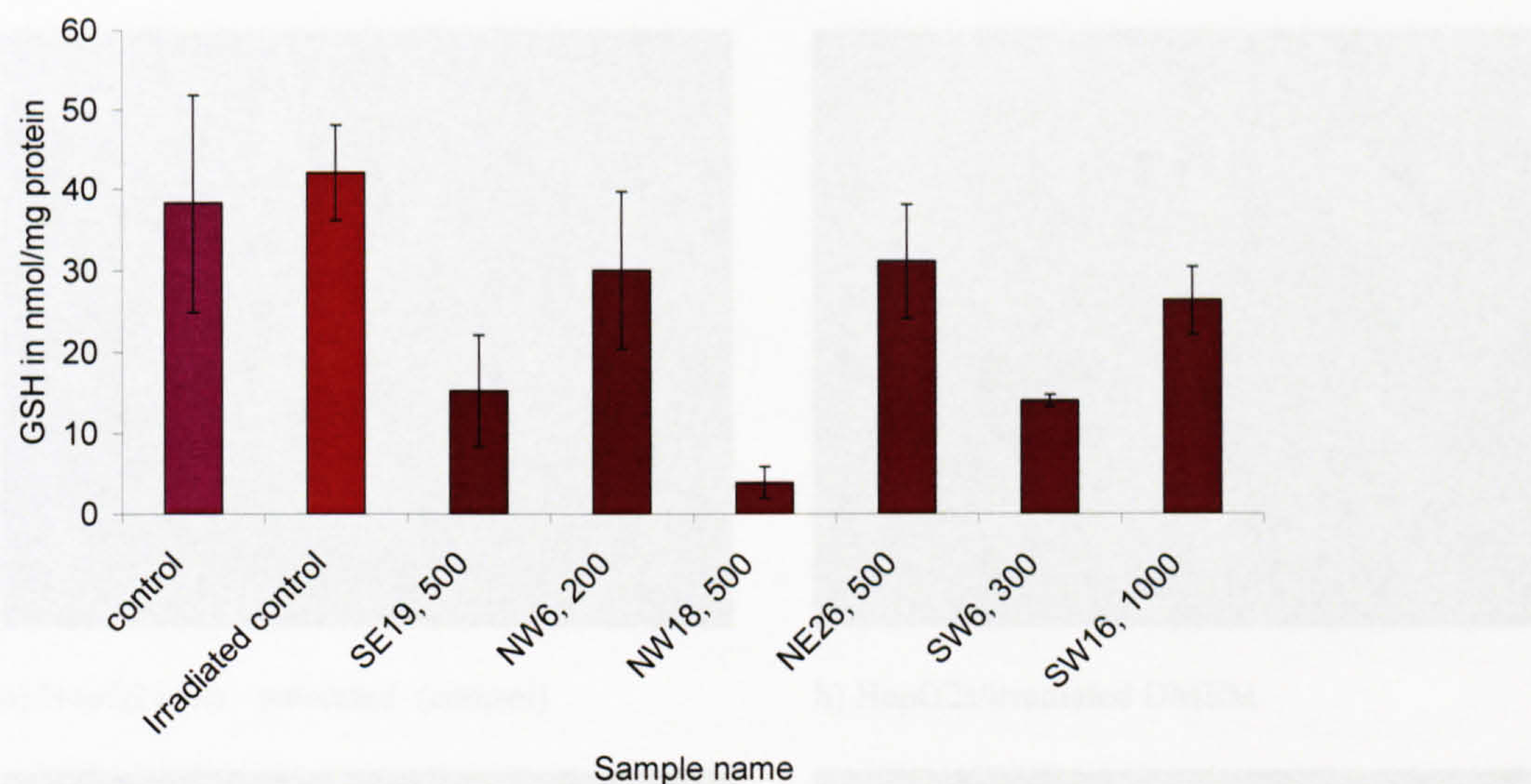
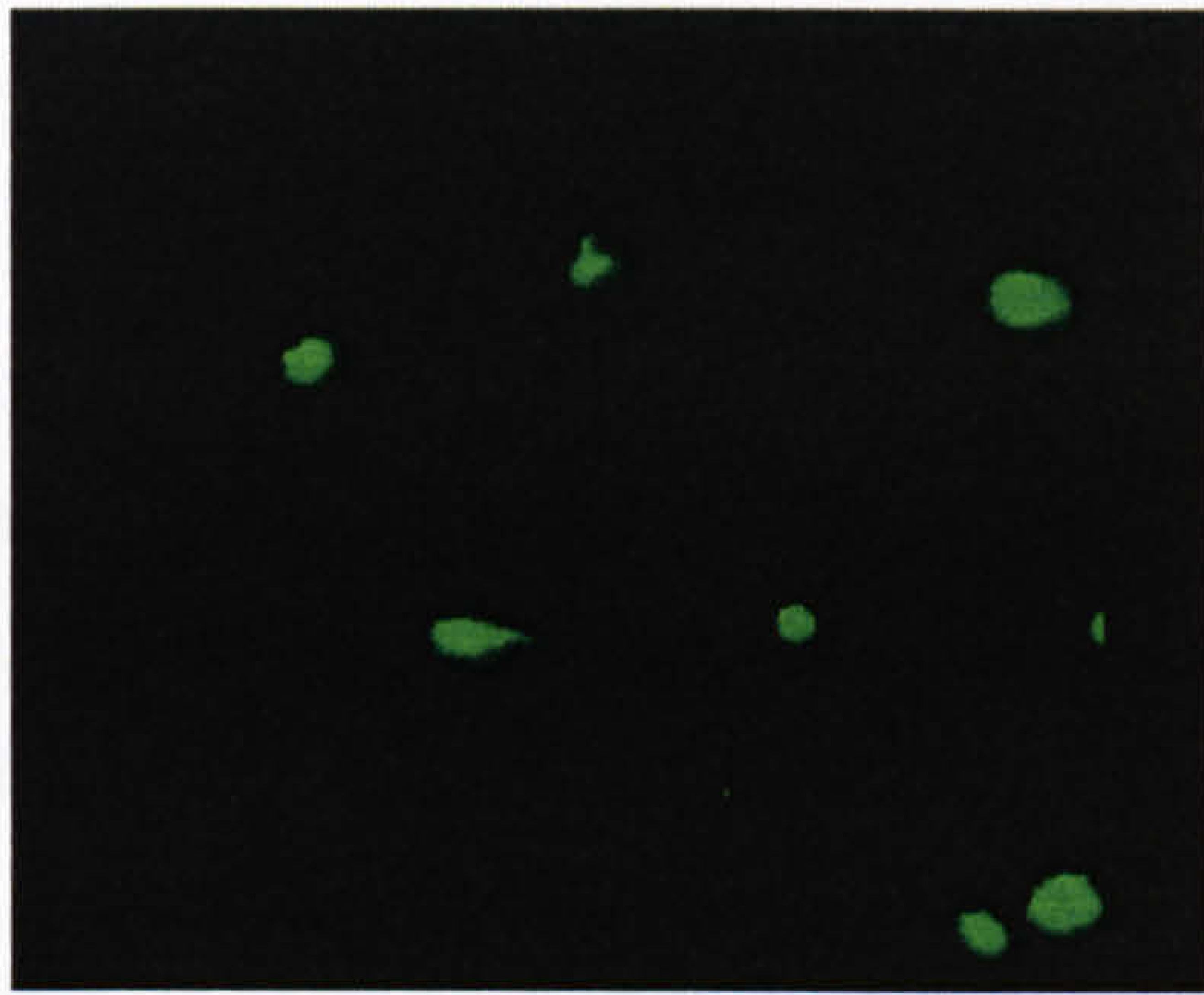


Figure 4.26 HepG2 cells exposed to soil/DMEM irradiated extracts for 24 hrs; reduced glutathione assay.

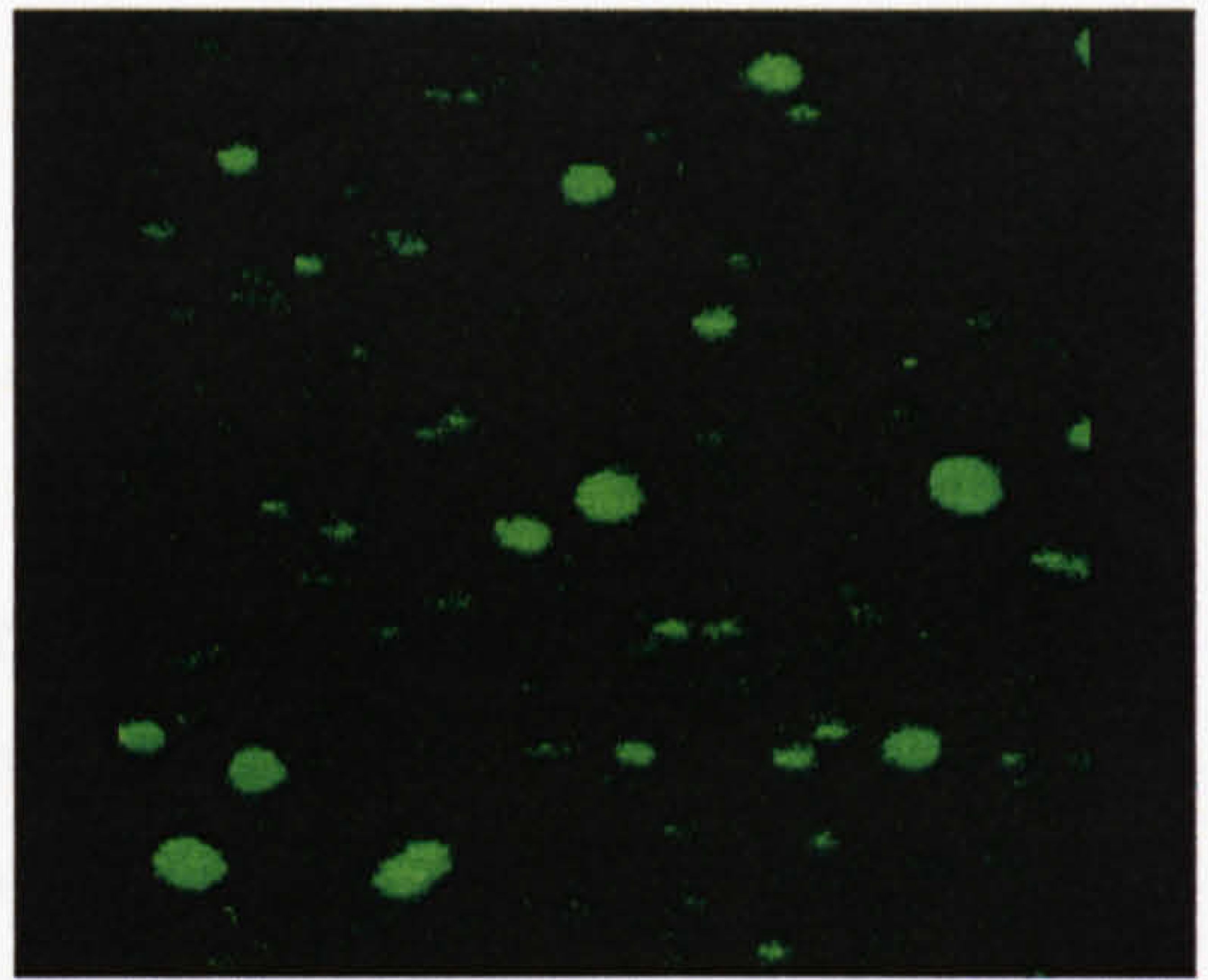
Results are expressed in nmol GSH/mg protein +/-SEM (n=3)

Comet images of HepG2 cells exposed to soil/DMEM extracts

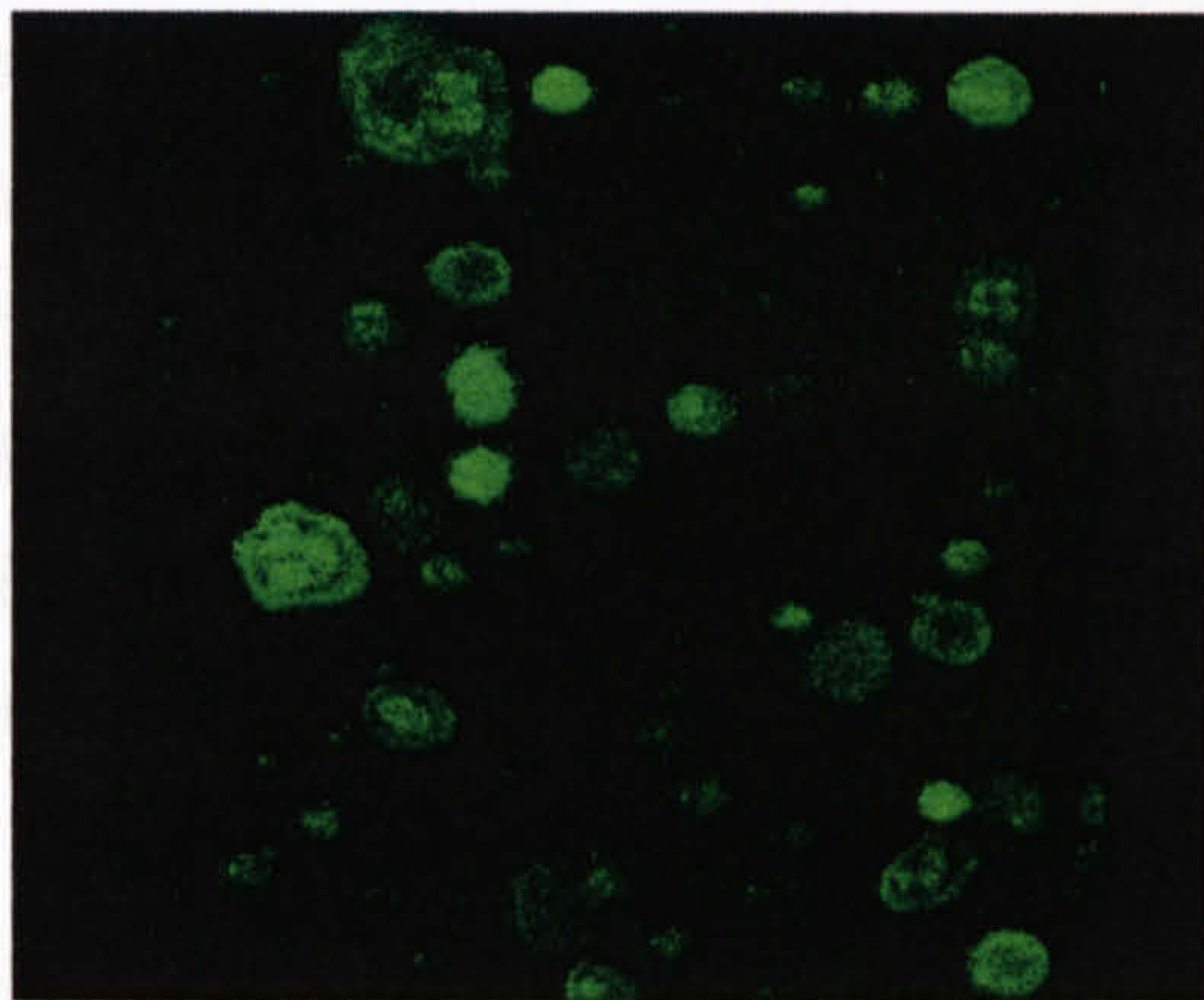
Figure 4.27 (a) shows "control" cells exposed to non-irradiated DMEM. Image (b) shows cells that had been exposed to γ-irradiated DMEM. The general debris (presumably DNA) makes analysis using comet software impossible. Images (c) and (d) show HepG2 cells exposed to 2 different soil/DMEM extract (γ-irradiated to sterilise), NE26,500 had low metal contamination and SW6,300 had high levels of metal contamination. Again the general appearance of the cells and the debris makes analysis using Comet software impossible.



a) HepG2 cells - untreated (control)



b) HepG2s/irradiated DMEM



c) NE26,500/DMEM irradiated

d) SW6,300/DMEM irradiated

Figure 4.27 Confocal microscope images of HepG2 cells exposed to a) un-irradiated DMEM b) irradiated DMEM c) NE26,500/DMEM irradiated and d) SW6,300/DMEM irradiated. Direction of electrophoresis was left to right

4.4 – DISCUSSION

4.4.1 Cu toxicity to HepG2 cells

The results of the four cytotoxicity assays (trypan blue, neutral red, MTT and reduced glutathione) showed that HepG2 cells exposed to Cu lost viability between 10 mg Cu/L and 50 mg Cu/L (equivalent to 157 μm and 787 μm). Comparing results in this thesis to other work, differences in toxicity responses of HepG2 cells to Cu have been seen. For example Roelofsen *et al.*, (2004) exposed HepG2 cells (grown in DMEM) to copper sulphate for 48hrs at what was described as a physiological concentration (0.5 μM) and a pathological concentration (100 μM); no reduction was seen in cell viability at these concentrations as assessed by the lactate dehydrogenase method. Urani *et al.* (2003) exposed HepG2 cells to 30 mg/L copper chloride for 24 hrs (as opposed to Cu nitrate used in this study) and examined the cell viability using the MTT assay and a total protein content method. Their results showed cell viability to be slightly below that of the control cells but within error limits (+/-20%) of normal viability. This would suggest the form or speciation of Cu the cells are exposed to may be fundamental to toxicity. Different types of human cells also respond differently; for example White & Cappai (2003) found that only 1.6 μM of extracellular Cu in culture medium (and depleted glutathione levels) led to neuronal cell death. Other factors present in solution will also affect Cu toxicity. For example, Singh *et al.* (2006) found that human peripheral blood mononuclear cells increased uptake of Cu into cells as Cu concentration rose, but not in the presence of Zn. Cu also caused a reduction in levels of GSH (LD50 of 115 μM) but with Zn present in solution the LD50 rose to 710 μM.

The results of the DNA damage experiments (comet assay) in this study showed a significant ($P<0.05$) increase between levels of DNA damage in control cells and those exposed even to the lowest levels of copper (0.1 mg/L). Again cell type and method of exposure appear to be important. O'Connor *et al.* (2003) found that *in vivo* oral supplementation of 3 and 6 mg/Cu/day (over 6 weeks) produced no alteration in levels of DNA damage in mononuclear lymphocytes and liver function was not adversely affected. Singh *et al.* (2006) found DNA fragmentation in human peripheral

blood mononuclear cells at 115 μM Cu. Conversely, Pan & Loo (2000) used the comet assay to assess the effect of Cu deficiency (by adding Cu chelator to growth medium) and found no DNA damage to lymphocytes but that antioxidant defence systems were compromised and made more susceptible to damage.

The interpretation of these results lies at the heart of any risk assessment of the genotoxicity of Cu and must include questions such as:- although the increase in DNA damage, as seen by the comet assay, at low levels of Cu is statistically significant, is it physiologically significant? Further work that looked at DNA damage and repair levels over time after single dose exposure and repeated doses may give some answers as to the long term toxic potential of Cu to human cells. The assessment of DNA damage caused by an excess (as opposed to a deficiency) of Cu, using the comet assay was a novel investigation.

4.4.2 Zinc toxicity to HepG2 cells

Zinc is also a micronutrient, essential to maintaining human health, and as such the majority of published literature has focused on the effects of zinc deficiency (e.g. Ho & Ames, 2002; Ho *et al.*, 2003) rather than over exposure. The results of this study show that HepG2 cells lost viability at a Zn concentration of approximately 10 mg/L. Comparing Cu and Zn on a molarity basis (see Tables 4.2 and 4.3) and mg/L shows Zn to be cytotoxic to HepG2 cells at a lower concentration. This was a surprising finding as Zn has been shown to protect human cells from the toxic effects of over exposure to Cu (Singh *et al.*, 2006) and mouse cells to over exposure to Cd (Fernandez *et al.*, 2003). Again, metal form/speciation may be the key to the level of toxic response. For example, Urani *et al.* (2003) dosed HepG2 cells with 50 mg/L zinc sulphate (regarded as an excessive level of exposure by the authors) over 24 hrs and using MTT and protein content assays, showed that cell viability dropped to between 60 and 80% of control cells. In this investigation, exposure to Zn nitrate at 50 mg/L, caused cell viability to drop to ~0%.

Results of the comet assay showed differences in levels of DNA damage (increases) between control and dosed cells were significant ($P>0.05$), but again, the importance of this difference can only be fairly judged by examining an extended time and dosing

regime. Banu *et al.* (2001) assessed the toxicity of Zn sulphate to mice and found that tail length decreased from 48 hrs post treatment onwards. Examination of the shape of the comet tails produced by Zn may supply some evidence as to the mechanism of DNA damage caused by Zn. The "hedgehog" shape of the tails, as opposed to the traditional tail seen in Cu treated cells, would indicate that the reduction in cell viability and consequent apoptosis induced at ~10 mg/L Zn, caused non-repairable nuclear fragmentation (Rundell *et al.*, 2003). The assessment of the toxicity of Zn to human cells using the comet assay was also a novel angle of research (as far as the author is aware).

4.4.3 Soil and soil extract toxicity to HepG2 cells

It appears that three key factors contributed to the exposure of contaminated soils to human cells yielding no quantitatively significant results; the specific cell line used (HepG2's), the properties of the soil extracts, and the inadvertent effect of sterilisation methods on the cell media which affected cell growth.

Selection of human tissue type

The robustness, or lack thereof, of HepG2 cells to withstand exposure to soil and soil extracts would suggest that this cell line is not appropriate as a soil toxicity indicator. Hartmann *et al.* (2003) and Tice *et al.* (2000) first and foremost recommend liver tissue for *in vivo* toxicity testing but also suggested the site-of-first-contact tissue i.e. gastrointestinal for orally administered substances, the respiratory tract or skin. So although cells of the GI tract do not absorb nutrients and toxicants, they may have the required "hardiness" to withstand whatever is immediately toxic to HepG2 cells, in the soil and soil extracts. Various human colon cell lines (e.g. Caco-2 cells Duthie & Collins, 1997) or intestinal cell lines (e.g. HCT-8) are available and may prove more suitable than HepG2 cells (although may still suffer the same problems as HepG2's).

Effect of soil and soil extracts

The actual soil and soil extracts, including the soil-less "control" extracts (other than soil/DMEM extracts) proved to be highly and almost immediately toxic to HepG2 cells. It may prove worthwhile to investigate adapting the soil bioavailability extracts to make the extract solution more suitable for use with human cells. For example, the BGS "intestinal" solution (see Chapter 3) may by adapted by removing the use of

digestive enzymes. It is unclear whether these enzymes have a definitive effect on metal extractability and if removed from the solution may potentially allow use with human cells as a soil toxicity assay?

Effect of sterilisation of soil/DMEM extracts on human cells

The conditions of culturing any human cell line require high levels of sterility to avoid contamination and out-competition of the human cells by bacteria or fungi. The levels of micro-fauna in soil require that soil extracts undergo sterilisation before contact with human cells. Heat sterilisation of DMEM was inappropriate (as vital media components would be denatured), filtering and steaming were inadequate leaving γ-irradiation as the only remaining option. Irradiation (sometimes referred to as cold sterilisation) is commonly (in the USA) used to sterilise food (reviewed in Smith & Pillai, 2004); doses of <10 KGy are considered "safe" – i.e. have destroyed microbial contamination without having altered the food. A dose of 20 KGy will eliminate the majority of soil bacteria (as reviewed in McNamara *et al.*, 2003) although doses of up to 70 KGy may be necessary to achieve total sterilisation. Although the results of the cytotoxicity assays suggested that HepG2's exposed to irradiated DMEM alone were viable, the phase contrast and comet assay pictures would contradict that conclusion. Anecdotal evidence may explain this effect (Dr Chris Jewell, personal communication). DMEM, when exposed to sunlight for prolonged time produces the same withered looking cells; UV radiation (from sunlight) of riboflavin and phenol red components in the medium leading to oxidative damage is thought to be responsible. Presumably the effect of the γ-irradiation is similar? The soil/DMEM extracts (irradiated) appear to provide an additional toxic insult to HepG2 cells, regardless of levels of extractable metal, suggesting some other compound extracted into solution (humic material?) was responsible.

4.4.4 Summary

HepG2 cells were found to be suitable for the detection of low, environmentally relevant levels of the heavy metals and environmental contaminants, Cu and Zn. Cu was found to cause cytotoxic damage to HepG2 cells at a concentration between 10 and 50 mg/L (157 μm and 787 μm) and to cause levels of DNA damage that were significantly different to untreated cells, at and above, 0.1 mg/L (1.5 μm). Further

assessment would be required in order to discover whether those levels cause a physiologically significant response.

Zn was found to be cytotoxic to HepG2 cells at around 10 mg/l (153 μm) and to cause DNA damage that was significantly different from control cells (similar to Cu), at and above, 0.1 mg/L (1.5 μm). Again, further investigation would be needed to show where physiologically significant effects would be caused. The results also showed Zn to be more cytotoxic to HepG2 cells, this was a surprising result as in studies examining Cu and Zn toxicity, Zn is usually found to have a protective action (Singh *et al.*, 2006).

The investigations into the toxicity of both Cu and Zn, (due to overexposure rather than deficiency) using the comet assay in this study were novel, especially in the context of contaminated land research. HepG2 cells were found to not be suitable for use in trying to determine whether metal contaminated soils were toxic, due to a range of problems in assay development (outlined above in 4.4.3). *In vitro* models, if capable of modelling *in vivo* responses, offer a potentially sensitive tool for inclusion in toxicity assessment including in the assessment of contaminated soils.

Chapter 5 – Development and optimisation of a bioassay using newly isolated luminescent *Pseudomonad* strains to determine the toxicity of contaminated soils

5.1 INTRODUCTION

Microbial bioassays are cheap, quick and reproducible compared to animal and plant or other multicellular organism toxicity tests and can be used either as screening tools, potential surrogates for more expensive test systems (usually humans/mammals) or as representatives of their natural ecosystems (i.e. the soil biota). Microbes are essential to soil functions and are therefore important components of soil toxicity assays.

Microbial bioassays are used as part of test batteries (e.g. Renoux *et al.*, 2001 and Tandy *et al.*, 2005) investigating soil quality or toxicity alongside, for example plants, worms and other species representative of the different soil trophic levels. They are commonly used in the assessment of contaminated land, both experimentally e.g. (Maxam *et al.*, 2000 and Ahtiainen *et al.*, 2002) and commercially (e.g. www.remedios.uk.com and www.cysense.com).

5.1.1 Microbial bioassays

Historically, microbial bioassays have an excellent pedigree in toxicology studies. The Ames/Salmonella histidine reversion assay (Ames *et al.*, 1973; Mortelmans & Zeiger, 2000) which tests the mutagenic potential (i.e. chronic damage) of chemicals was one of the first used in toxicity assessment and is still used regularly (especially as an integral part of international pharmaceutical testing protocols of genotoxicity (ICH, 1995; 1997). The test is fully adaptable to use with soils (Courty *et al.*, 2004; Watanabe *et al.*, 2005), the disadvantages being that the assay only tests for chronic toxicity, requires external addition of a metabolic activation system (usually rat S9 microsomal fraction) and takes up to 72 hrs to perform.

5.1.2 Luminescent microbial bioassays

As important as chronic toxicity testing is, there is also a need to investigate whether chemicals/contaminated soils can cause acute toxic responses (i.e. poisoning and/or death); microbial bioassays are widely used as acute toxicity indicators. Increasingly popular are the "light" assays, i.e. luminescence and/or fluorescence, with toxicity shown by either an increase ("lights on") or decrease ("lights off" e.g. Microtox system) in light levels compared with a control. The major advantages of "light system" bioassays are that they are non-destructive and real time monitoring is possible.

One of the most widely used (and one of the first luminescent or "lux" based) is the Microtox® (Azur Environmental) system. Based on a naturally luminescent marine bacterium, *Vibrio fischeri*, Microtox® has been used to assess hundreds of different chemicals, mixtures and soil and sediment extracts. For example PAH mixture toxicity was investigated by Haeseler *et al.*, (1999), TNT and RDX contaminated soil toxicity (before and after treatment) by Rocheleau *et al.*, (1999), quinolone toxicity, singly and as mixtures by Backhaus *et al.*, (2000), heavy metals and arsenic toxicity in aqueous solutions at pH 6 and 7 by Fulladosa *et al.*, (2005) and metal contaminated soil leachate before and after remediation by Calace *et al.*, (2005). The main disadvantages of the Microtox® bioassay are that *Vibrio fischeri* is a marine organism that requires consistently high saline concentrations, neutral pH (which may alter metal speciation) and may not be relevant when assessing the toxicity of soils and soil extracts or leachates.

The advent of genetic manipulation i.e. insertion of the lux operon into a new organism, either by transcriptional fusion into the chromosome or on a plasmid, has allowed for the development of a wide range of "lux" organisms coupled either to metabolic pathways, to give an overall toxicity response or a response to specific indicator genes. For example, Min *et al.*, (2003), used five recombinant *Escherichia coli* strains that had lux genes coupled to promoters specific for DNA damage, membrane damage, oxidative damage, protein damage and general toxicity to examine the effects of dioxins. The range of biosensors

(microbial and protein based) specific for heavy metals is substantial and reviewed in Verma & Singh (2005).

As with the Microtox® assay, *E.coli* and many other organisms commonly used as microbial toxicity assays examining contaminated soil (extracts/leachates) are not native soil organisms and therefore not strictly ecologically relevant. Soil organisms that have been lux engineered include *Rhizobium leguminosarum* (Reid *et al.*, 1998) but more commonly used are strains of *Pseudomonas*. For example, Sinclair *et al.* (1999) used a *Pseudomonas fluorescens* to examine DCP toxicity; Jacob *et al.* (2001) used *P. fluorescens* and *P. putida* to assess oil bioremediation; Paton *et al.* (1995) used *P. fluorescens* to assess the bioavailability of a range of heavy metals and Petanen & Romantschuk, (2003) used two *P. fluorescens* to investigate toxicity and bioavailability of mercury and arsenic contaminated soil.

Stauber & Davies (2000) make the point that selection of appropriately sensitive test species and experimental endpoints and protocols are needed to ensure relevance of any assay used. A key problem with microbial bioassays is that there is very little consistency in the academic literature as to experimental protocols being used. Factors such as the length of time an assay is run for (what time point is used to judge an effect), what liquid (diluent) is used to resuspend the organism in and the point of insertion of the lux gene into an organism all vary with very little (if any) explanation or justification given as to why. In this thesis a *Pseudomonas* species (native soil organism) which was isolated and manipulated (two different lux gene insertions were made) by Gorres, (2001) was made available for use, not having previously been optimised for use with soils or heavy metals. (NB these strains were also chosen for use as they were available at no cost).

5.1.3 Experimental aims

Therefore the aims of this work were:-

1. To develop and optimise a luminescent Pseudomonas bioassay as a soil toxicity indicator, specifically examining several areas of methodology where there has been little investigation to date including:-

a) The effect of calculating results over time (by integration), compared to use of a specific time point to assess toxicity.
b) The diluent solution used to resuspend the bacteria and whether it modified luminescent behaviour.
c) Whether location of the lux gene within the genome showed different results using 2 different lux gene insertion locations into the same *Pseudomonas*.

2. Examine the toxicity of the Byker soils using a variety of soil extract solutions, towards the *Pseudomonads*. To date, no record can be found of simulated human physiological extracts being used in conjunction with a microbial assay (all others use water, buffer or solvent extracts).

3. And finally to compare the biological response of the microbes to the known chemical data (i.e. extractable metal) to see if any correlations could be drawn.

5.2 MATERIALS AND METHODS

5.2.1 Provenance of bioassay bacterial test strains

Two genetically modified luminescent *Pseudomonad* strains were gifted from the laboratory of Prof A.G. O'Donnell having been isolated and manipulated by Dr Heike Gorres (Gorres, 2001). Originally isolated from contaminated soil (former tar works, St Anthony's, Newcastle upon Tyne), a strain named CN1/12 was mated with an *E.coli* S17/1 lambda pir harbouring a pUT miniTn5 construct (Winson *et al.*, 1998). The promoterless luxCDABE operon from the insect pathogen *Photorhabdus luminescens* was then inserted into the CN1/12 chromosome by random mutagenesis. Transconjugants were selected for using kanamycin and Lux+ phenotypes (i.e. constitutive luminescence expression) were then screened for in the dark. The two strains used in this study were referred to as *lux1* and *lux3*. Neither strain had been previously standardised for use with metals or soils.

5.2.2 *Growth and maintenance conditions of luminescent bacteria*

Both organisms were grown up in Luria-Bertani (LB) medium (Sigma);10 g tryptone, 5 g yeast extract and 10 g NaCl was made up to 1 L with dH_2O and sterilised by autoclaving (121°C and 15 psi for 20 mins). 2 ml/L kanamycin (Kan) solution antibiotic (Sigma) was added once the solution had cooled.

Bacterial stock were maintained either frozen (-80°C) or on agar plates at 4°C. Frozen stock was prepared using 1.5 ml of dense bacterial culture and 0.5 ml sterile 60% w/v glycerol solution, frozen down at -20°C overnight and then maintained at -80°C. Cells were revived by thawing quickly and pipetting and spreading 200 µl onto agar plates (LB as above, plus 15 g/L agar powder – Lab M), grown up for 2 days at 27°C and then maintained at 4°C for a maximum of 1 month.

Cell cultures were grown in 250 ml conical flasks (autoclaved to sterilise and plugged with cotton wool) using a single colony starter in 100 ml LB+Kan at 27°C and 120 rpm in an orbital shaker (Sanyo Gallenkamp) and harvested at ~18 hrs (mid-log phase) at an OD_{600} of 2 determined using a Kontron uvikon 930 spectrophotometer. An OD_{600} of 2 was equivalent to $\sim 6.4 \times 10^8$ CFU/ml.

5.2.3 *General solutions used*

Six solutions (diluents) were used to resuspend the Pseudomonads; distilled water (dH_2O), LB (as above), DMEM (Gibco- see 4.2.1), 0.1 M 4-Morpholinoethanesulphonic acid hydrate (MES) pH 6 (Sigma), ¼ strength Ringers solution (Merck, 2 tablets/L dH_2O) and 0.1 M KCl (Sigma). Four metal solutions were used; copper ($Cu(NO_3).3H_2O$), nickel ($Ni(NO_3)_2.6H_2O$), lead ($Pb(NO_3)_2$) and zinc ($Zn(NO_3)_2.4H_2O$). All were supplied by BDH as 1000 mg/L stock solutions. Dilutions were made using dH_2O and pH alteration made as required using NaOH and HCl.

5.2.4 *Bioassay experimental conditions*

Cells were grown as described in section *5.2.2* and 10 ml of cell suspension was transferred to a 50 ml centrifuge tube. The suspension was centrifuged at 6000 x

g for 10 mins and the supernatant then discarded. The appropriate diluent (10 ml) was then added and the cell pellet was resuspended by shaking. This was centrifuged at 6000 x g for 10 mins, the supernatant discarded and the cell pellet finally resuspended in 10 ml of the appropriate diluent. Opaque (white) 96-well assay plates were loaded with 180 µl of "test solution" (metal solution or soil extract). *Lux1* or *lux3* (20 µl) was added per well (this proportion of 9:1 used by Sinclair *et al.*, 1999) using a multichannel pipette and luminescence read using a Thermo Labsystems Fluoroskan FL machine with Thermo Labsystems Fluoroskan Ascent Software. Luminescence levels were measured once a minute for 30 mins with results expressed as relative light units (RLU). Diluent and water were used as blanks (0% luminescence) and lux/diluent as a control (100% luminescence). All test solutions, blanks and controls were performed in triplicate (n=3). Sensitivity of both lux strains, resuspended in the 6 diluents, to increasing concentrations of Cu, Ni, Pb and Zn was investigated. The range of metal concentrations chosen (0-10 mg/L) was determined by the levels of metal that were found to be extractable from Byker soils shown in Chap. 3 (original results) and Table 5.1.

Table 5.1 Levels of metal found in various soil extracts

	Cu in mg/L			Ni in mg/L			Pb in mg/L			Zn in mg/L		
Sample name	H_2O	BGS Int1	DMEM	H_2O	BGS Int1	DMEM	H_2O	BGS Int1	DMEM	H_2O	BGS Int1	DMEM
SW12, 600	0.02	0.14	0.29	n.d.	0.02	0.07	n.d.	0.08	0.08	0.03	0.11	n.d.
SW14, 700	0.05	0.20	0.65	0.00	0.01	0.04	0.01	0.08	0.11	0.01	0.13	n.d.
SW16, 1000	0.05	0.19	0.23	n.d.	0.02	0.07	0.02	0.05	0.23	0.06	0.09	n.d.
SE18, 500	0.01	0.06	0.22	0.03	0.01	0.06	0.06	n.d.	0.12	0.02	n.d.	n.d.
NE8, 200	0.07	0.21	0.69	n.d.	0.01	0.04	0.02	0.04	0.07	0.05	0.16	n.d.
NE26, 500	0.02	0.08	0.25	n.d.	0.01	0.01	0.01	0.02	0.10	0.03	0.01	n.d.
NE45, 1000	0.04	0.21	0.64	0.00	0.01	0.05	0.01	0.03	0.06	0.02	0.07	n.d.
NW30, 1000	0.05	0.20	0.59	0.00	0.01	0.05	0.00	0.04	n.d.	0.02	0.14	n.d.
NW18, 500	0.11	0.35	0.94	n.d.	0.03	0.11	n.d.	0.20	0.04	0.34	1.74	1.51
SE10, 300	0.01	0.14	0.94	0.01	0.04	0.05	n.d.	0.01	0.06	0.00	0.47	0.10
SE19, 500	0.14	1.36	3.67	n.d.	0.01	0.04	0.01	0.26	0.04	0.07	0.69	0.06
SW6, 300	0.22	4.09	13.96	0.00	0.05	0.08	0.00	0.22	0.03	0.10	3.15	0.65
NE27, 500	0.05	0.33	0.84	0.00	0.02	0.06	n.d.	0.10	0.04	0.13	0.27	0.03
NW6, 200	0.07	0.85	4.73	0.01	0.03	0.11	0.02	0.03	0.06	0.03	0.29	0.01
SE30, 1000	0.10	0.41	1.00	0.00	0.02	0.04	0.01	0.03	0.05	0.03	0.25	n.d.
SW10, 500	0.08	0.32	0.84	n.d.	0.01	0.03	0.05	0.06	0.10	0.06	0.36	0.12

Cu, Ni, Pb and Zn in mg/L found in soil/water extracts, BGS Int1 fraction of a soil/simulated gut extract and soil/DMEM extract from Byker soils. n.d. = not detected. Values over 1mg/L are highlighted in yellow.

The main range of metals extracted in the different solutions lay between 0 and 10 mg/L with the majority of samples <1 mg/L. As such, the concentrations of metals that were chosen investigating sensitivity of the Pseudomonads were 0, 0.01, 0.05, 0.1, 0.3, 0.5, 0.7, 0.9, 1, 2, 5 and 10 mg/L. Lux response to soil extracts (water, simulated gut and DMEM) was also then examined using selected diluents. The "Int1" fraction of the BGS simulated gut extract was chosen to represent the simulated gut extracts (preliminary experiments showed the low pH of the "Stom" fraction killed the *Pseudomonads*).

5.2.5 Measurement of luminescence, data gathering and interpretation

Levels of luminescence were measured kinetically over a 30 minute period. Traditionally, results of experiments investigating toxicity using luminescence involves leaving bacteria and test sample to equilibrate (anywhere from 5 mins- e.g. Gorres, 2001 to 60 mins e.g. Min *et al.*, 2003) and measuring luminescence at a specific time point. Advances in detection capability now allow measurements over time (kinetic) to be taken. As the luminescence of controls is not steady over time (see Figure 5.1) and time taken to load a 96-well plate (up to 5 mins) can vary, examining luminescence at one particular time point can lead to error in data interpretation. Therefore in this study, results were calculated by summing luminescence readings taken over 30 mins, taking the mean of triplicate readings and expressing as a percentage of the control (control=100%).

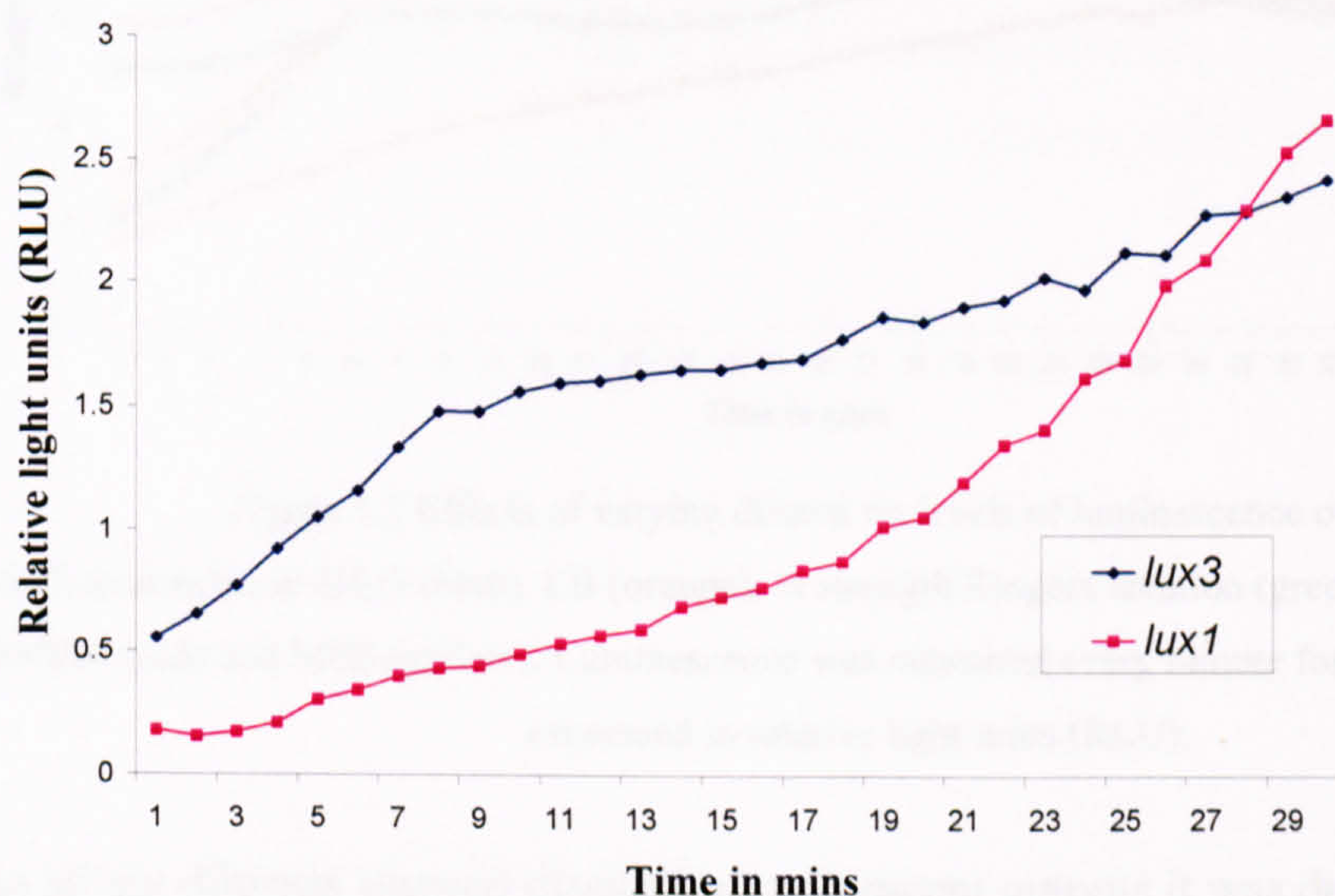


Figure 5.2 - Luminescence of *lux1* (pink) and *lux3* (blue) in relative light units (RLU) over time (30 mins).

Statistical analysis

MINITAB® 14 Statistical software for Windows was used to perform regression analysis of the luminescence data against metal bioavailability and basic soil parameter data in order to investigate which, if any, parameter most influenced luminescence levels (i.e. produced a toxic response).

5.3 RESULTS

5.3.1 Standardisation of experimental conditions: time and pH

As seen in Figure 5.1 (method section), the two *Pseudomonads* luminescent output varied between organisms and over time. Here, Figure 5.2 shows a typical graph of luminescence varying depending on what diluent the bacteria had been suspended in (and over time).

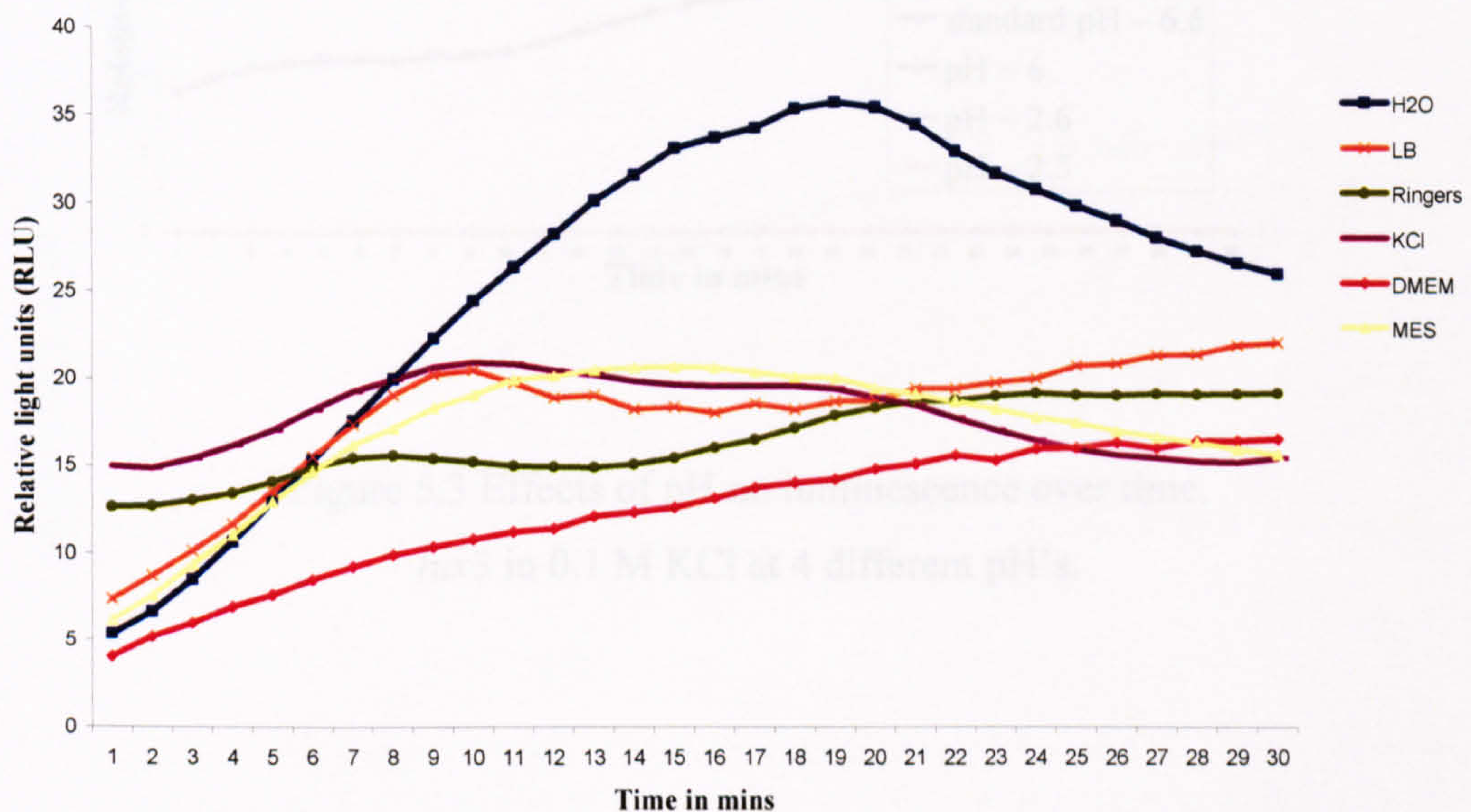


Figure 5.2 Effects of varying diluent on levels of luminescence over time.
*lux*3 suspended in dH_2O (blue), LB (orange), ¼ strength Ringers solution (green), 0.1 MKCl (purple), DMEM (red) and MES (yellow). Luminescence was measured every minute for 30 minutes and results expressed in relative light units (RLU).

As all six diluents showed dissimilar luminescent outputs it was decided to investigate the response to metals and soil extracts with all six.

pH has also been shown to be an important consideration (Sinclair 1999) when using luminescent bacteria as toxicity indicators. Figures 5.3 and 5.4 show typical examples of changes in luminescence by *lux*3 with reductions in pH; KCl and H_2O as diluents are used as examples. In order to avoid pH being responsible for variation in luminescence levels, all metal solutions were pH adjusted to pH 5.5.

Figures 5.5 and 5.6 show how the choice of which time point to read luminescence levels can give very different results; summation of the data of the time period can reduce this problem.

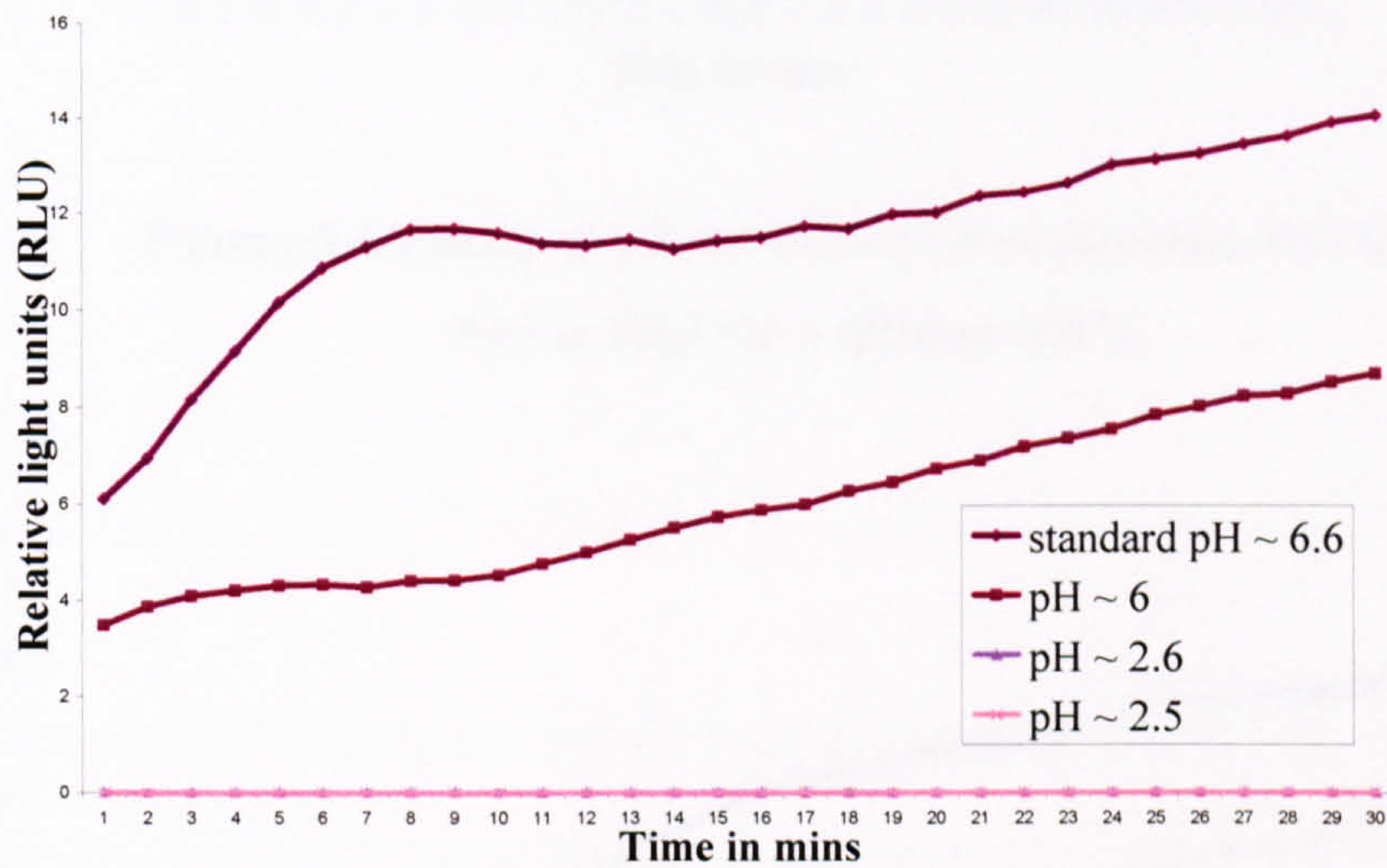
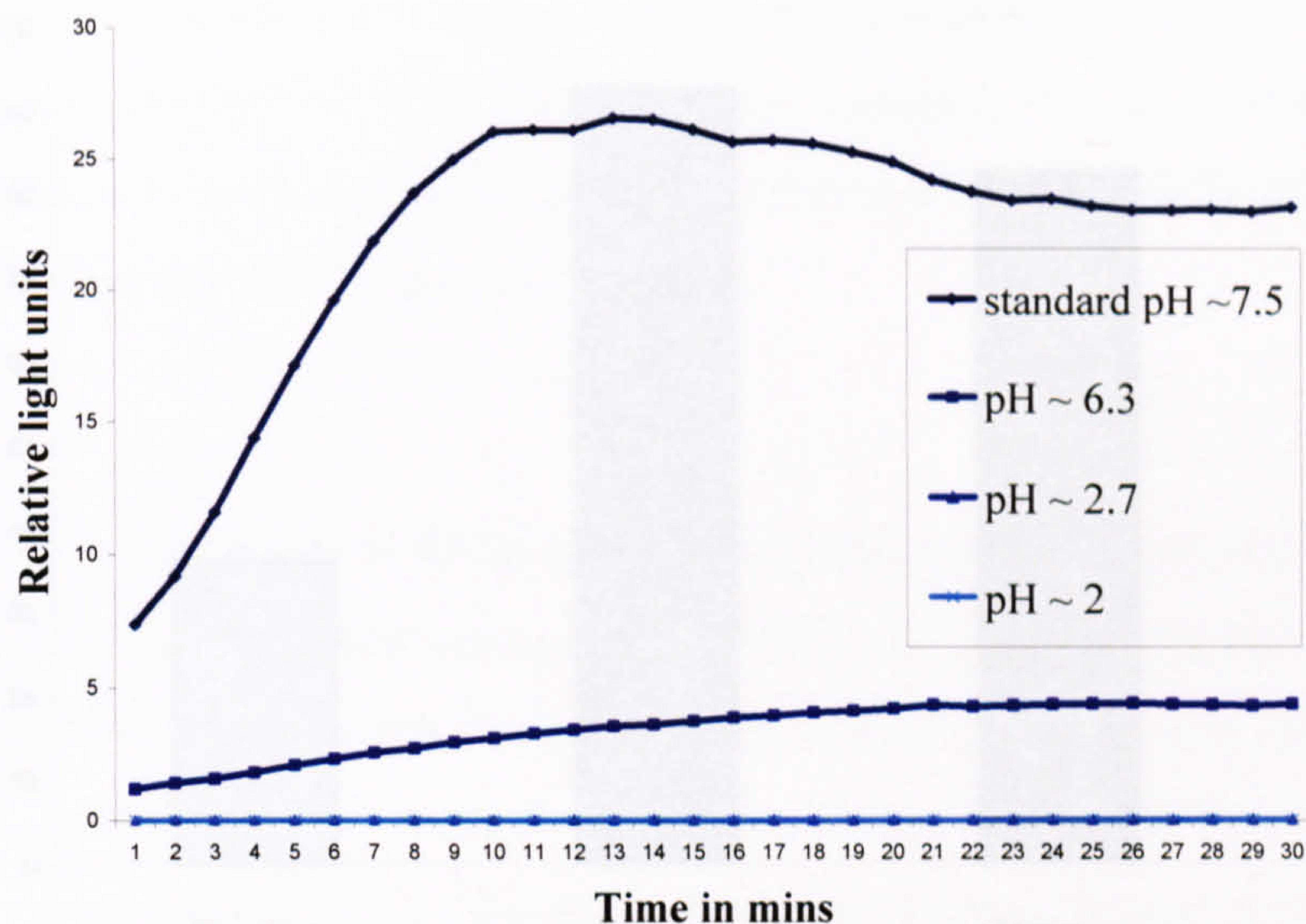


Figure 5.3 Effects of pH on luminescence over time.
*lux*3 in 0.1 M KCl at 4 different pH's.



Figures 5.4 Effects of pH on levels of luminescence over time

*lux*3 in dH_2O at 4 different pH's.

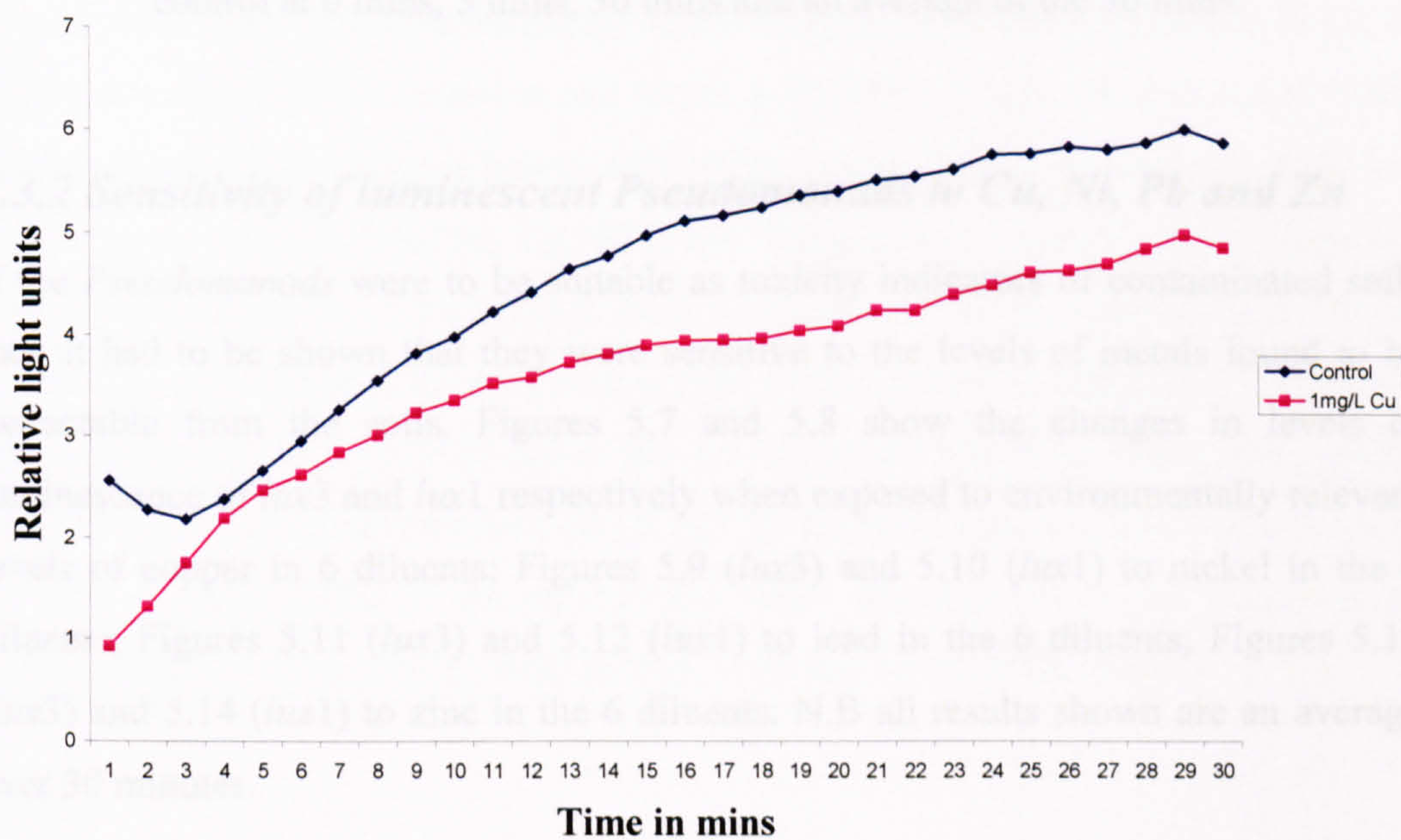


Figure 5.5 Luminescence in relative light units of *lux3* resuspended in ¼ strength Ringers solution; "control" and 1 mg/L Cu samples shown.

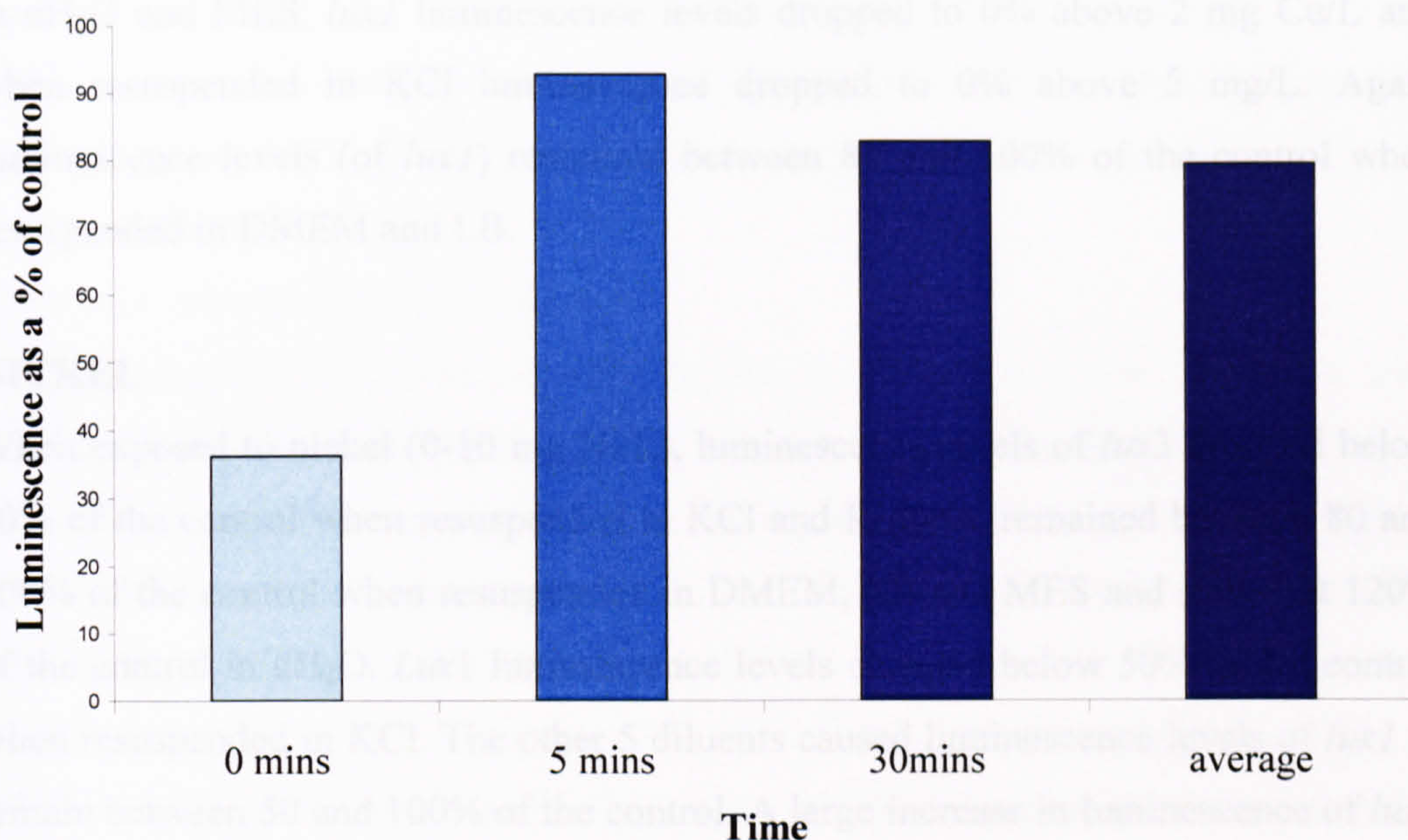


Figure 5.6 Levels of luminescence of the 1 mg/L Cu sample as a percentage of the control at 0 mins, 5 mins, 30 mins and an average of the 30 mins.

5.3.2 Sensitivity of luminescent Pseudomonads to Cu, Ni, Pb and Zn

If the *Pseudomonads* were to be suitable as toxicity indicators of contaminated soils then it had to be shown that they were sensitive to the levels of metals found to be extractable from the soils. Figures 5.7 and 5.8 show the changes in levels of luminescence of *lux*3 and *lux*1 respectively when exposed to environmentally relevant levels of copper in 6 diluents; Figures 5.9 (*lux*3) and 5.10 (*lux*1) to nickel in the 6 diluents; Figures 5.11 (*lux*3) and 5.12 (*lux*1) to lead in the 6 diluents; Figures 5.13 (*lux*3) and 5.14 (*lux*1) to zinc in the 6 diluents. N.B all results shown are an average over 30 minutes.

COPPER

When exposed to copper (above 1 mg Cu/L) the luminescence of the *lux*3 strain dropped to 0% of the control value when resuspended in the following diluents: KCl, Ringers, dH_2O and MES. Luminescence levels remained between 80 and 100% of the control in DMEM and LB up to 10mg Cu/L. Above 1 mg Cu/L, when resuspended in Ringers, levels of luminescence of the *lux1* strain dropped to 0%. When resuspended

in dH_2O and MES, *lux1* luminescence levels dropped to 0% above 2 mg Cu/L and when resuspended in KCl luminescence dropped to 0% above 5 mg/L. Again luminescence levels (of *lux1*) remained between 80 and 100% of the control when resuspended in DMEM and LB.

NICKEL

When exposed to nickel (0-10 mg Ni/L), luminescence levels of *lux*3 dropped below 50% of the control when resuspended in KCl and Ringers; remained between 80 and 100% of the control when resuspended in DMEM, LB and MES and stayed at 120% of the control in dH_2O. *Lux*1 luminescence levels dropped below 50% of the control when resuspended in KCl. The other 5 diluents caused luminescence levels of *lux1* to remain between 50 and 100% of the control. A large increase in luminescence of *lux1* (a "spike") was seen at 1 mg Ni/L in when resuspended in dH_2O and Ringers.

LEAD

Exposure to lead caused luminescence levels of *lux*3, when resuspended in dH_2O, to drop below 20% of the control at and above 2 mg Pb/L. When resuspended in MES, luminescence levels dropped below 20% of the control at and above 5 mg Pb/L and when resuspended in DMEM, LB, KCl and Ringers luminescence levels remained between 80 and 110% of the control. *Lux*1 luminescence levels, when resuspended in dH_2O, KCl and Ringers, dropped below 50% of the control at and above 2 mg Pb/L and when resuspended in MES levels fell at and above 5 mg Pb/L. When resuspended in DMEM, at all concentrations (0-10 mg Pb/L) luminescence levels (*lux1*) were between 80 and 100% of the control and when resuspended in LB (again at all concentrations 0-10 mg Pb/L) were between 100 and 140% of the control.

ZINC

Exposure of *lux*3 to Zn, when resuspended in KCl, caused luminescence levels to drop below 20% of the control by 5 mg Zn/L. When resuspended in DMEM, luminescence levels dropped below 30% of the control by 5 mg Zn/L and when resuspended in dH_2O luminescence levelled off at 50% at and above 2 mg Zn/L. When resuspended in Ringers luminescence decreased to ~40% of the control, in MES levelled off at ~ 70% of the control (from 1 mg Zn/L) and in LB luminescence levels decreased to ~60% of the control. Levels of luminescence of strain l*ux*1 dropped below 20% when

resuspended in KCL (by 1 mg Zn/L), in DMEM and Ringers below 50% (by 5 mg Zn/L), in MES and LB remained between 50 and 100% and in dH_2O luminescence levels dropped to 75% at 5 mg Zn/L and then rose to 160% at 10 mg Zn/L.

Summary of diluent/strain responses

Exposure to the four different metal solutions in a range of diluents caused a wide variation in luminescent response from both *Pseudomonad* strains (*lux3* and *lux1*). However there was no consistent effect and neither organism could be described as being more sensitive to metals than the other. Therefore both bacteria were used to investigate the response to the Byker soil extracts.

Of the six diluents that *lux3* and *lux1* were resuspended in and exposed to the 4 metals, overall in DMEM and LB both organisms appeared to exhibit less sensitivity than in the other four diluents. Of the remaining four diluents, KCl and dH_2O were selected to investigate the sensitivity of the two bacteria to the Byker soil extracts as the *Pseudomonad* strains appeared to have greater sensitivity to the metals in those diluents.

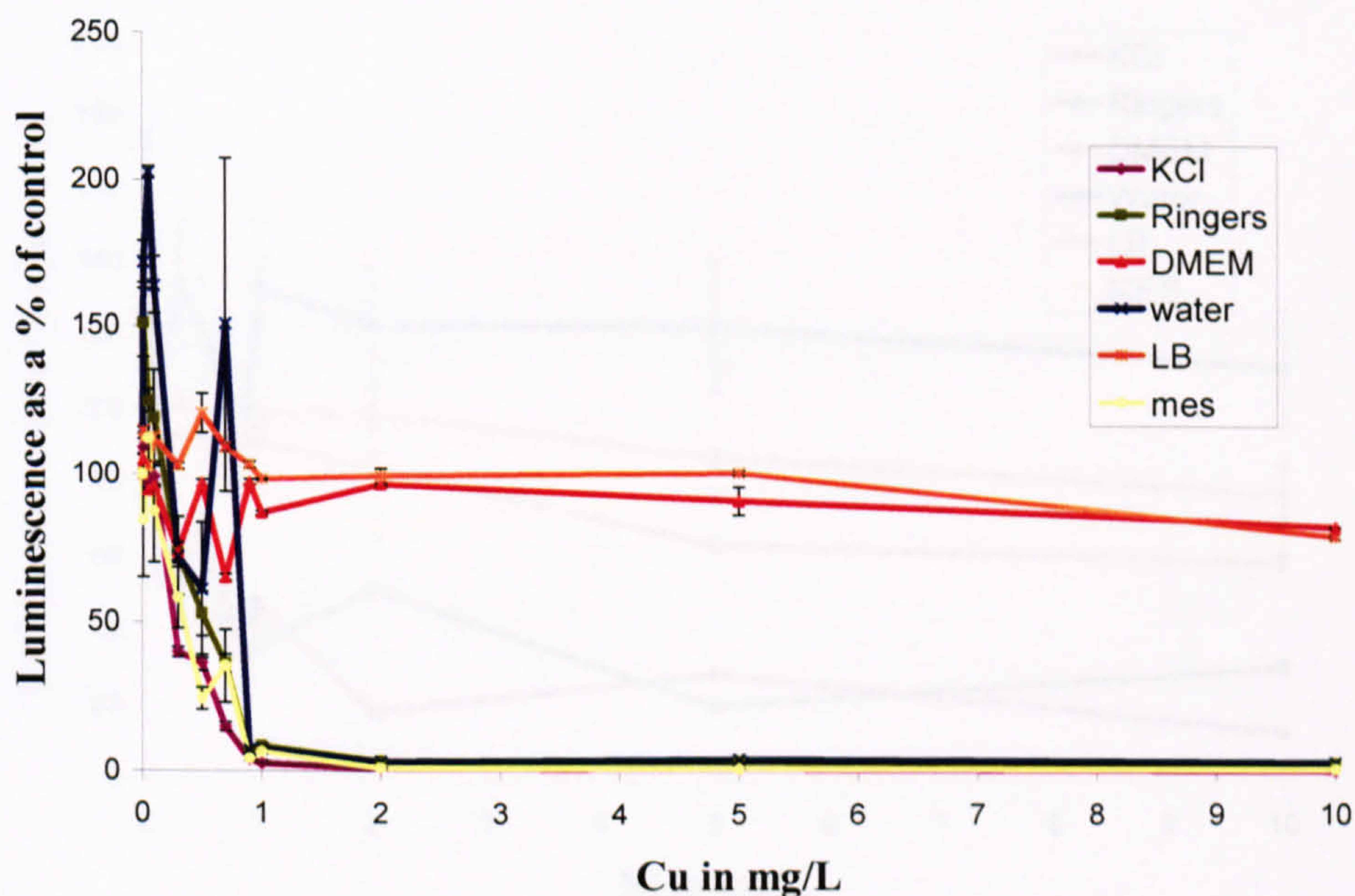


Figure 5.7 Changes in luminescence levels of *lux*3, exposed to 0-10 mg/L Cu. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux3 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES (yellow).

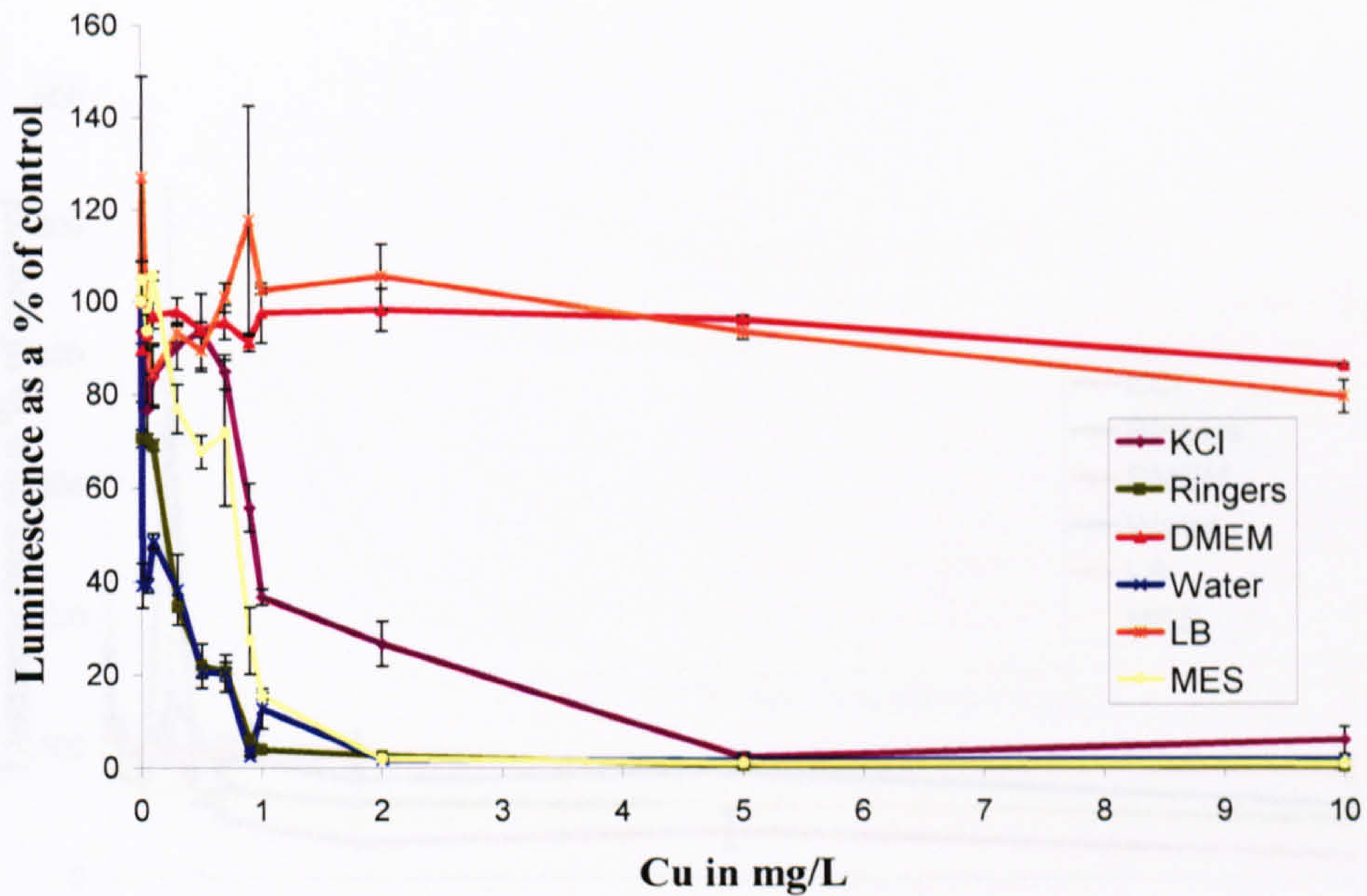


Figure 5.8 Changes in luminescence levels of *lux*1, exposed to 0-10 mg/L Cu. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux1 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES (yellow).

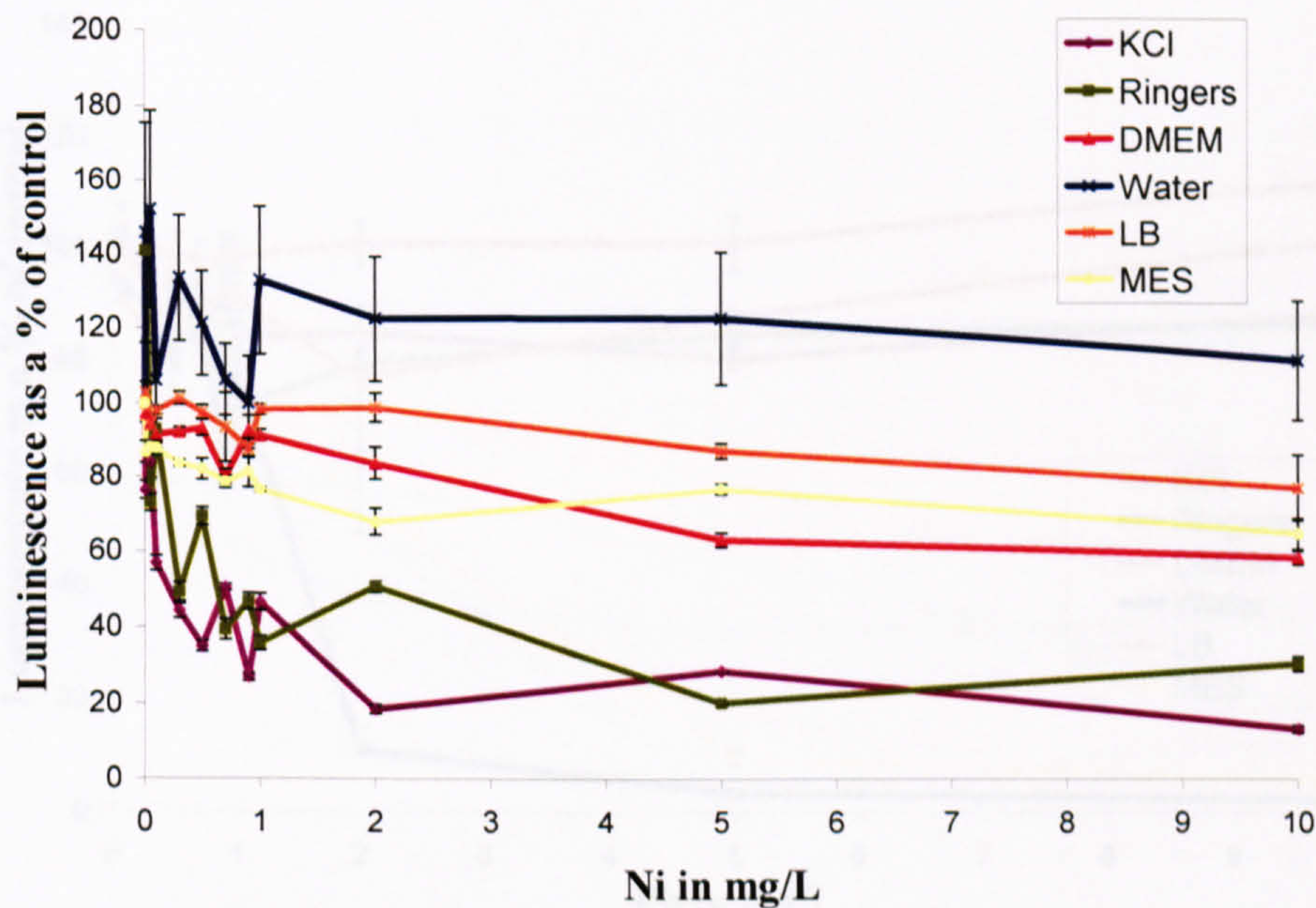


Figure 5.9 Changes in luminescence levels of *lux*3, exposed to 0-10 mg/L Ni. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux3 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES (yellow).

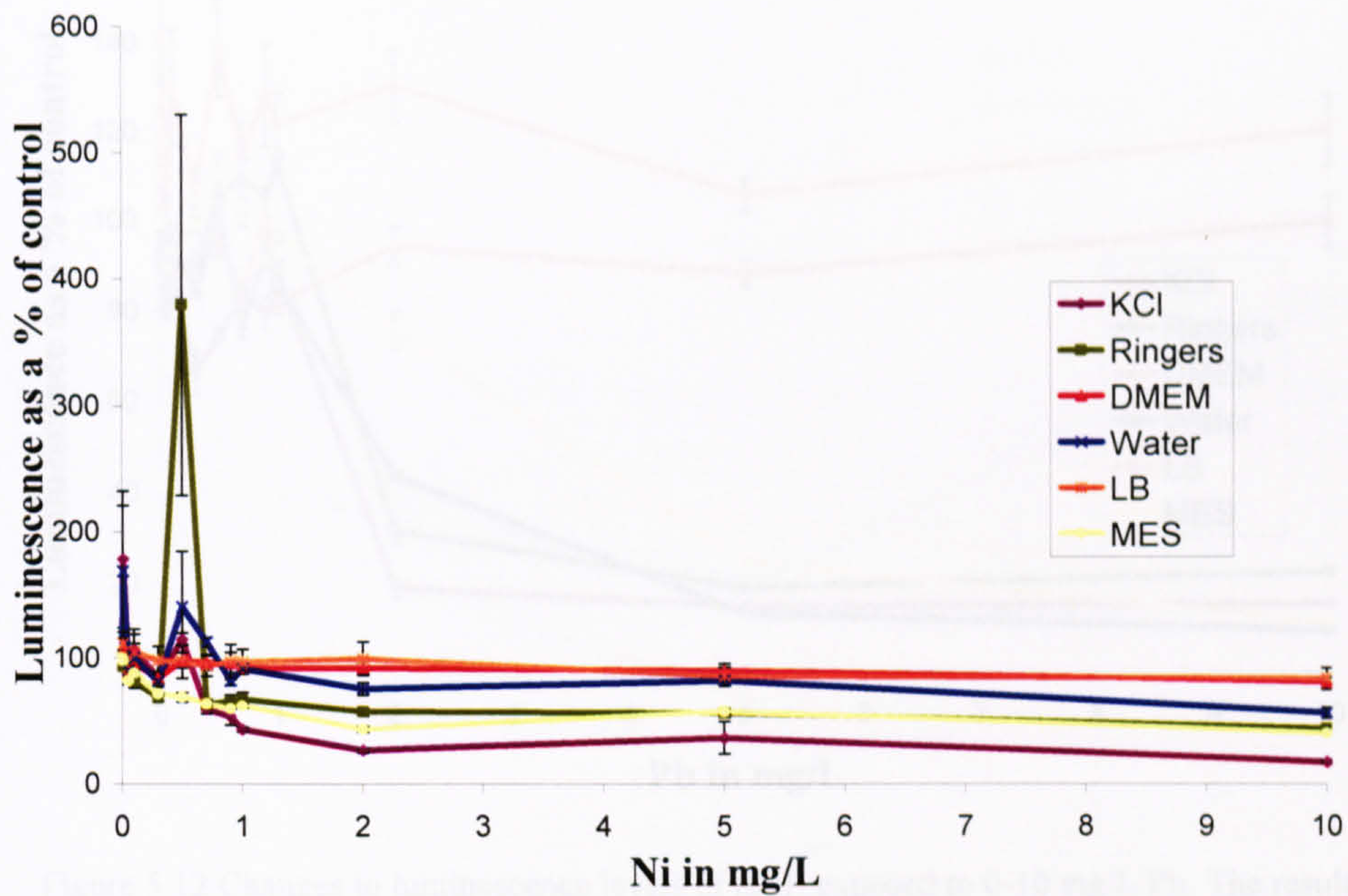


Figure 5.10 Changes in luminescence levels of *lux*1, exposed to 0-10 mg/L Ni. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux1 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES (yellow).

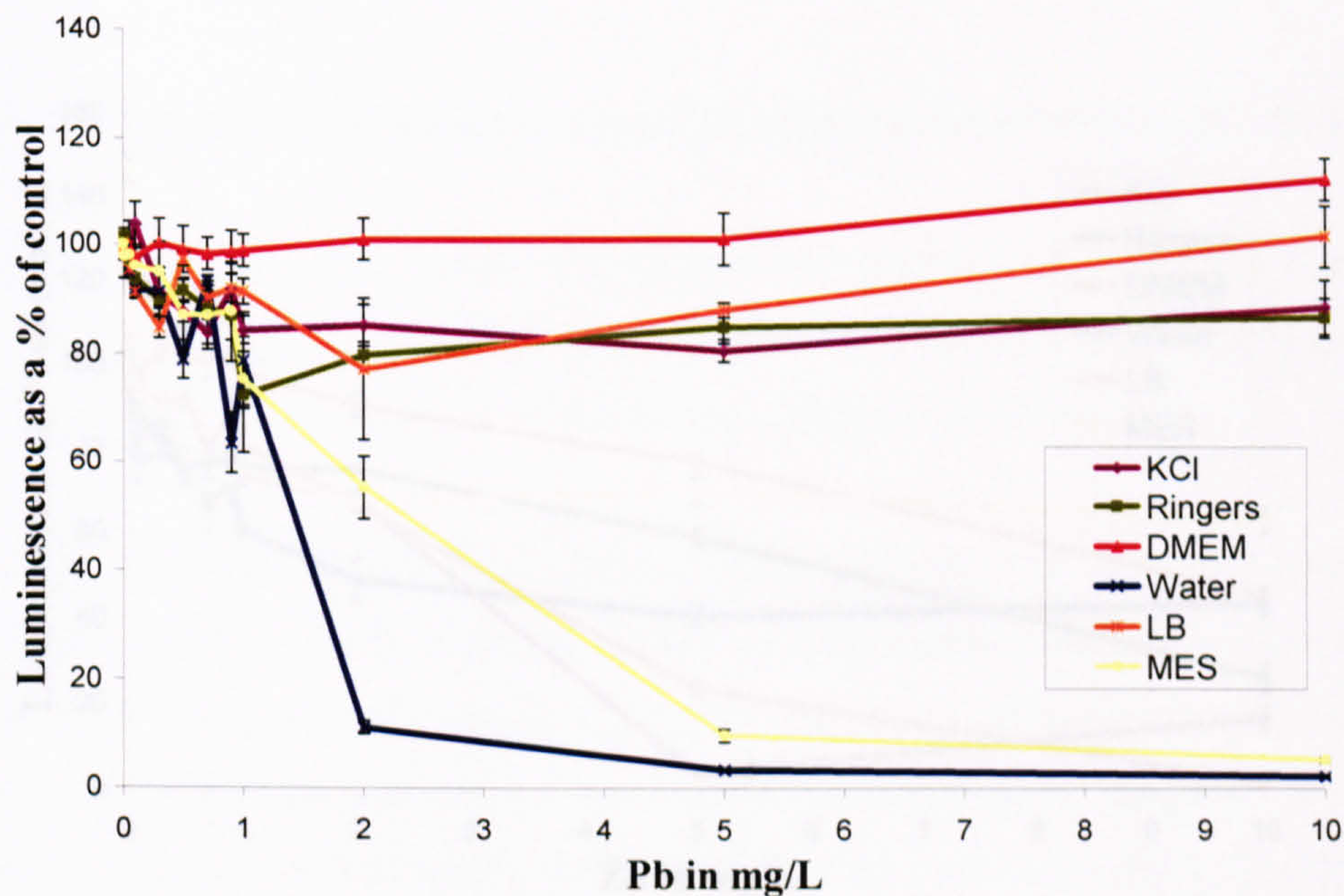


Figure 5.11 Changes in luminescence levels of *lux*3, exposed to 0-10 mg/L Pb. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux3 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES (yellow).

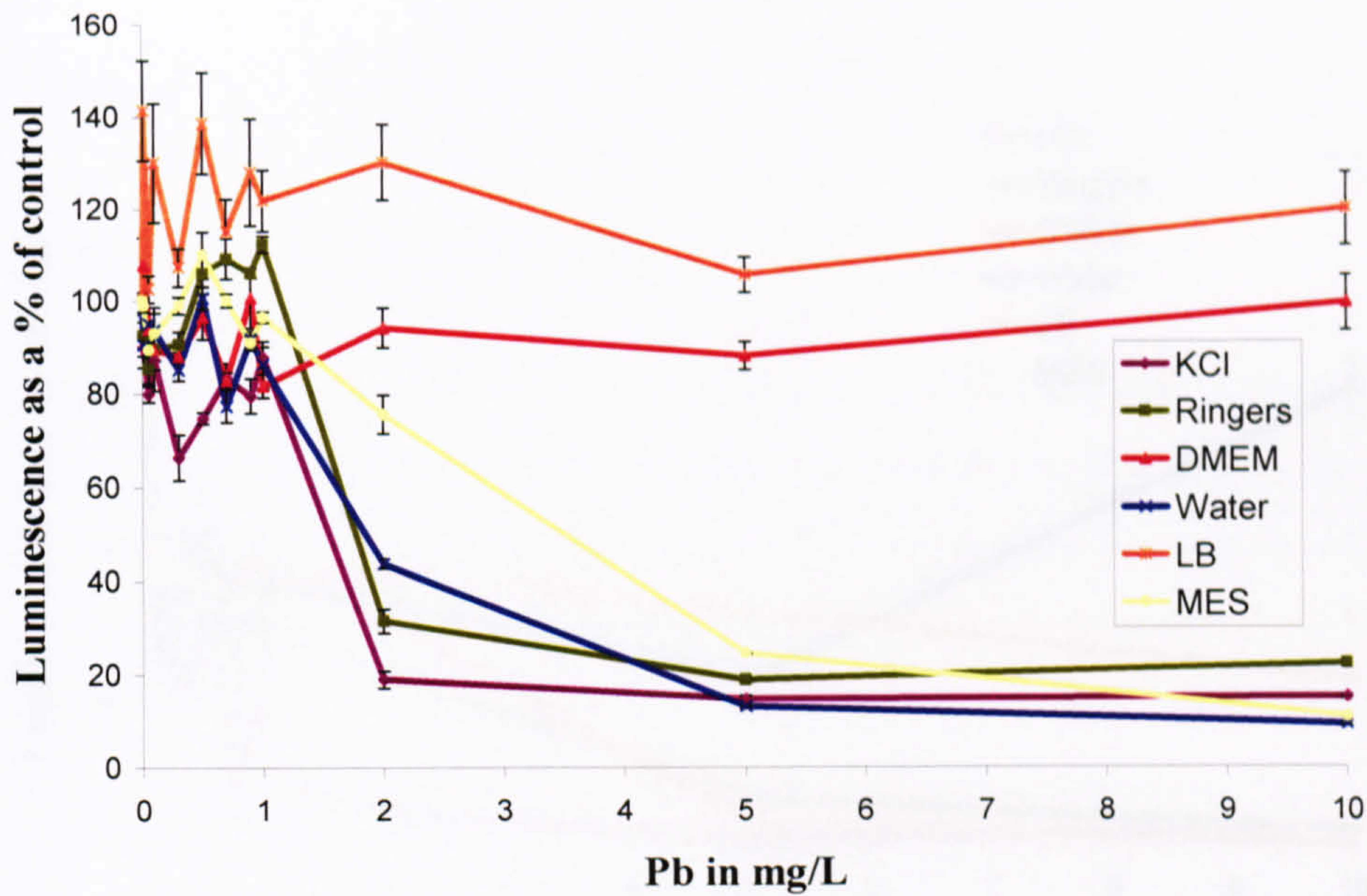


Figure 5.12 Changes in luminescence levels of *lux*1, exposed to 0-10 mg/L Pb. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux1 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES

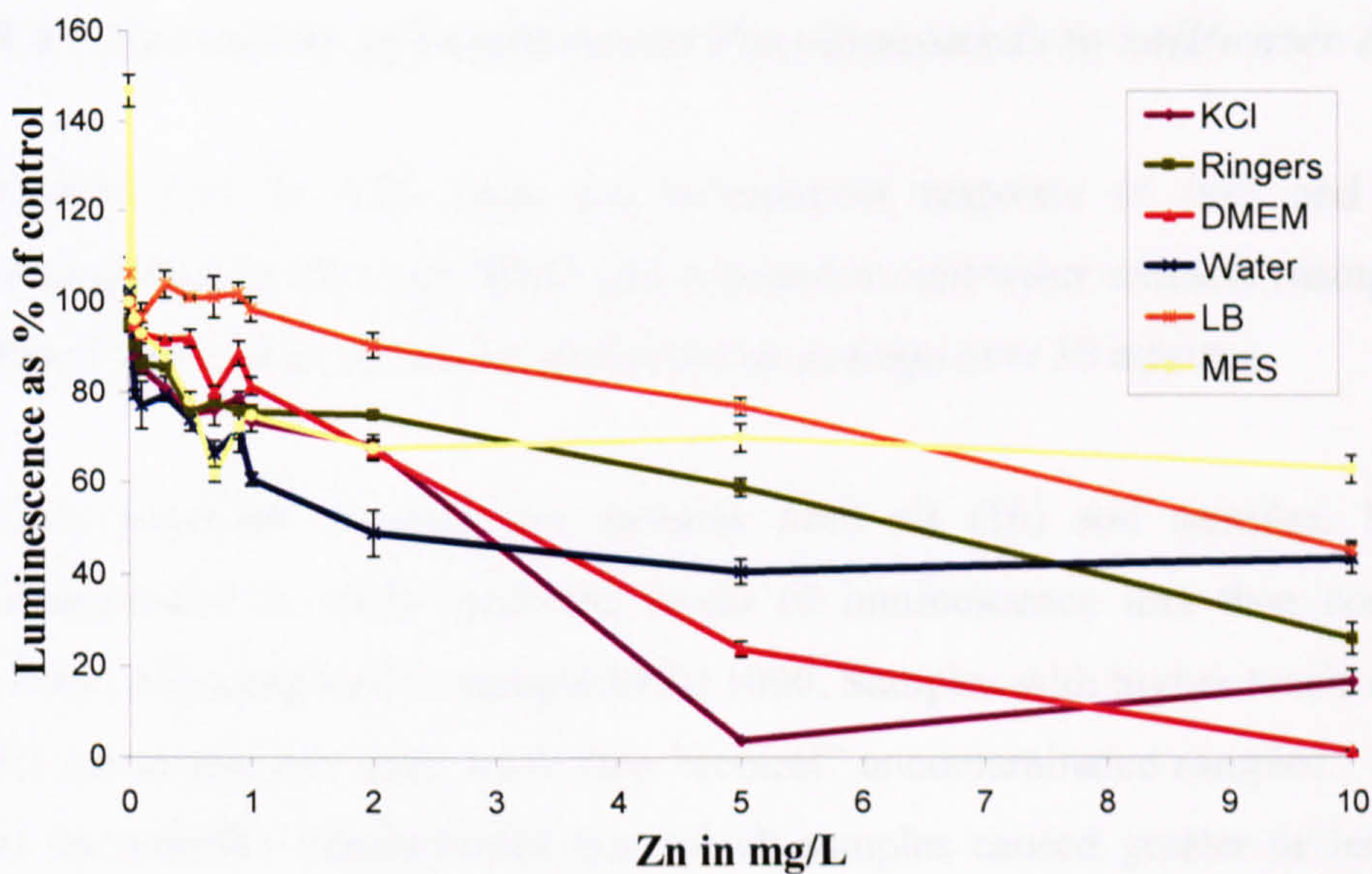


Figure 5.13 Changes in luminescence levels of *lux*3, exposed to 0-10 mg/L Zn. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux3 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES (yellow).

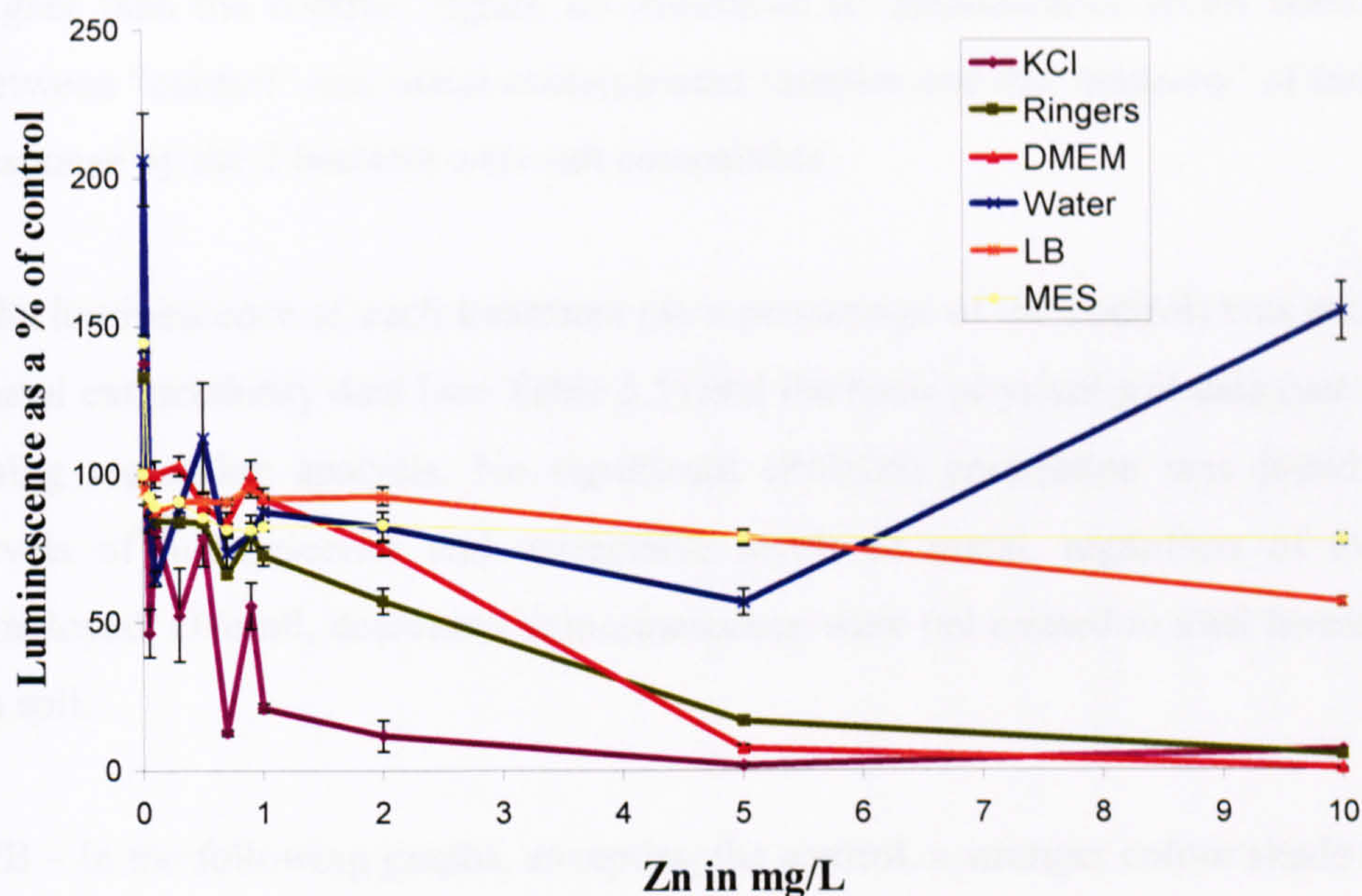


Figure 5.14 Changes in luminescence levels of *lux*1, exposed to 0-10 mg/L Zn. The results are an average over 30 mins and are expressed as a percentage of the control (100%) +/- SEM (n=3). Lux1 was suspended in 0.1 MKCl (purple), ¼ strength Ringers solution (green), DMEM (red), dH_2O (blue), LB (orange) and MES (yellow).

5.3.3 Sensitivity of luminescent Pseudomonads to soil/water extracts

Figures 5.15 to 5.18 show the luminescent response of *lux*3 and *lux*1 strains resuspended in KCl and dH_2O and exposed to soil/water extracts (using 16 selected Byker soils). N.B. all results shown are an average over 30 minutes.

Upon exposure to soil/water extracts from all (16) soil samples, both bacteria (resuspended in KCl) exhibited levels of luminescence less than control (100%) except when exposed to sample SE30,1000. Samples with higher levels of total metal did not appear any more toxic than "control" uncontaminated samples. The "pattern" of the samples luminescence (i.e. which samples caused greater or lesser levels of luminescence) was not similar for *lux3* and *lux1*. Neither strain appeared to be more sensitive to the soil/water extracts.

When resuspended in dH_2O, all the samples caused levels of luminescence that were higher than the control. Again, no difference in luminescence levels could be seen between "control" and metal contaminated samples and the "patterns" of luminescent response by the 2 bacteria were not comparable.

The luminescence of each treatment (as a percentage of the control) was compared to metal extractability data (see Table 5.1) and the basic physical soil data (see chapter 3) using regression analysis. No significant ($P<0.05$) connection was found between levels of luminescence and extractable levels of metal, regardless of the diluent employed. Overall, decreases in luminescence were not related to total levels of metal in soil.

NB – In the following graphs, excepting the control, a stronger colour shade is used to indicate higher levels of "total" metal in the sample

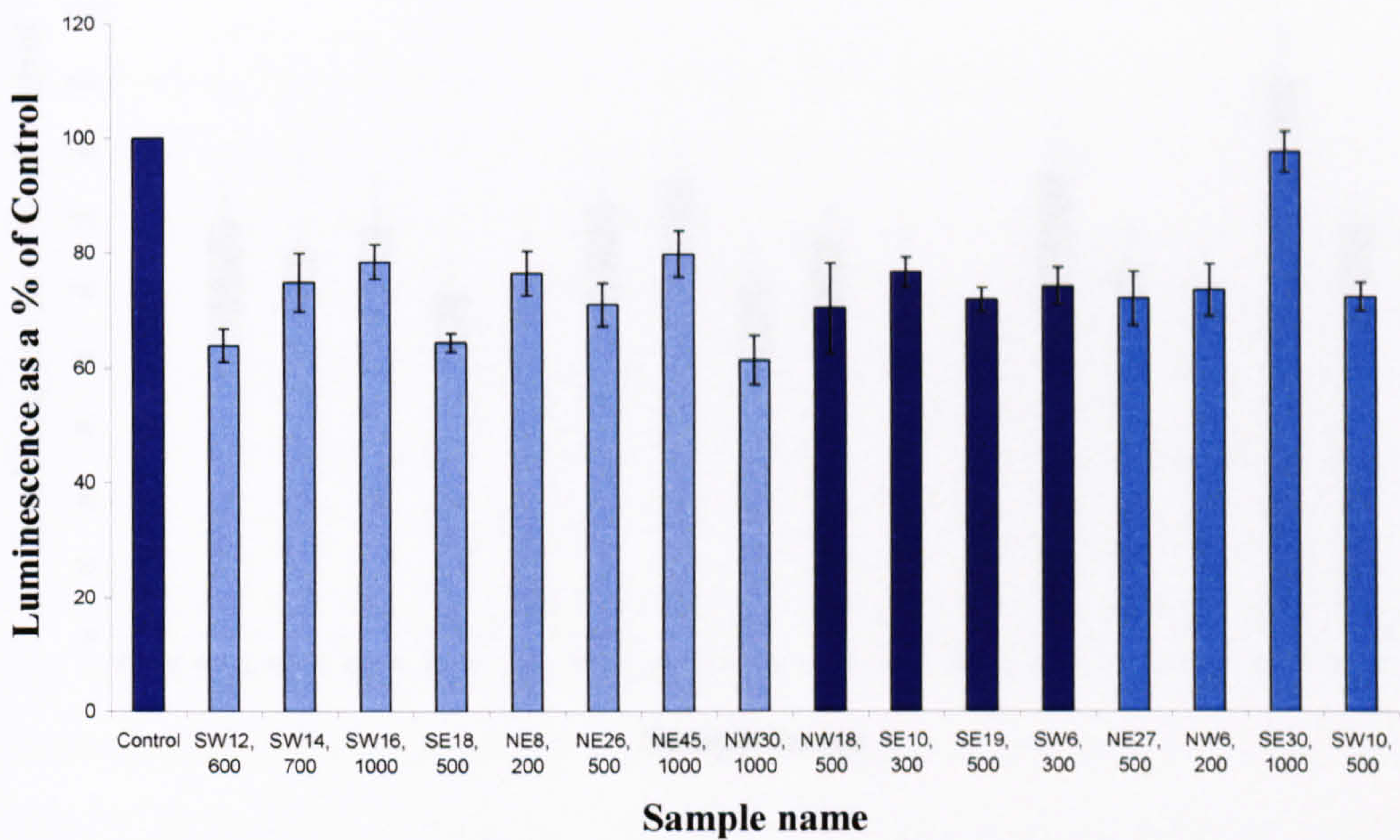


Figure 5.15 Variation in luminescent response of *lux3* exposed to soil/water extracts from urban soil samples. 0.1 M KCL used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3.

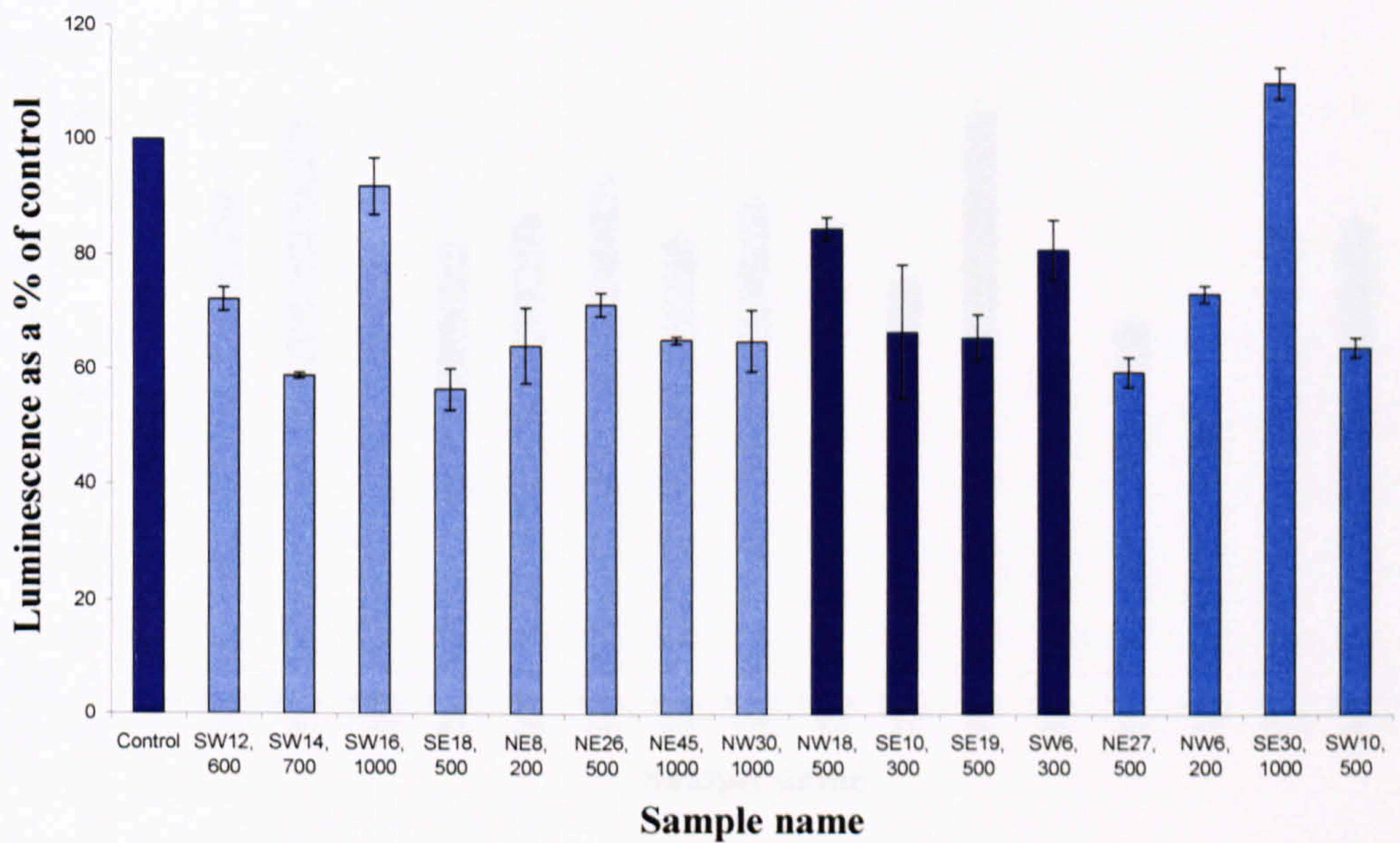


Figure 5.16 Variation in luminescent response of *lux1* exposed to soil/water extracts from urban soil samples. 0.1 M KCL used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3.

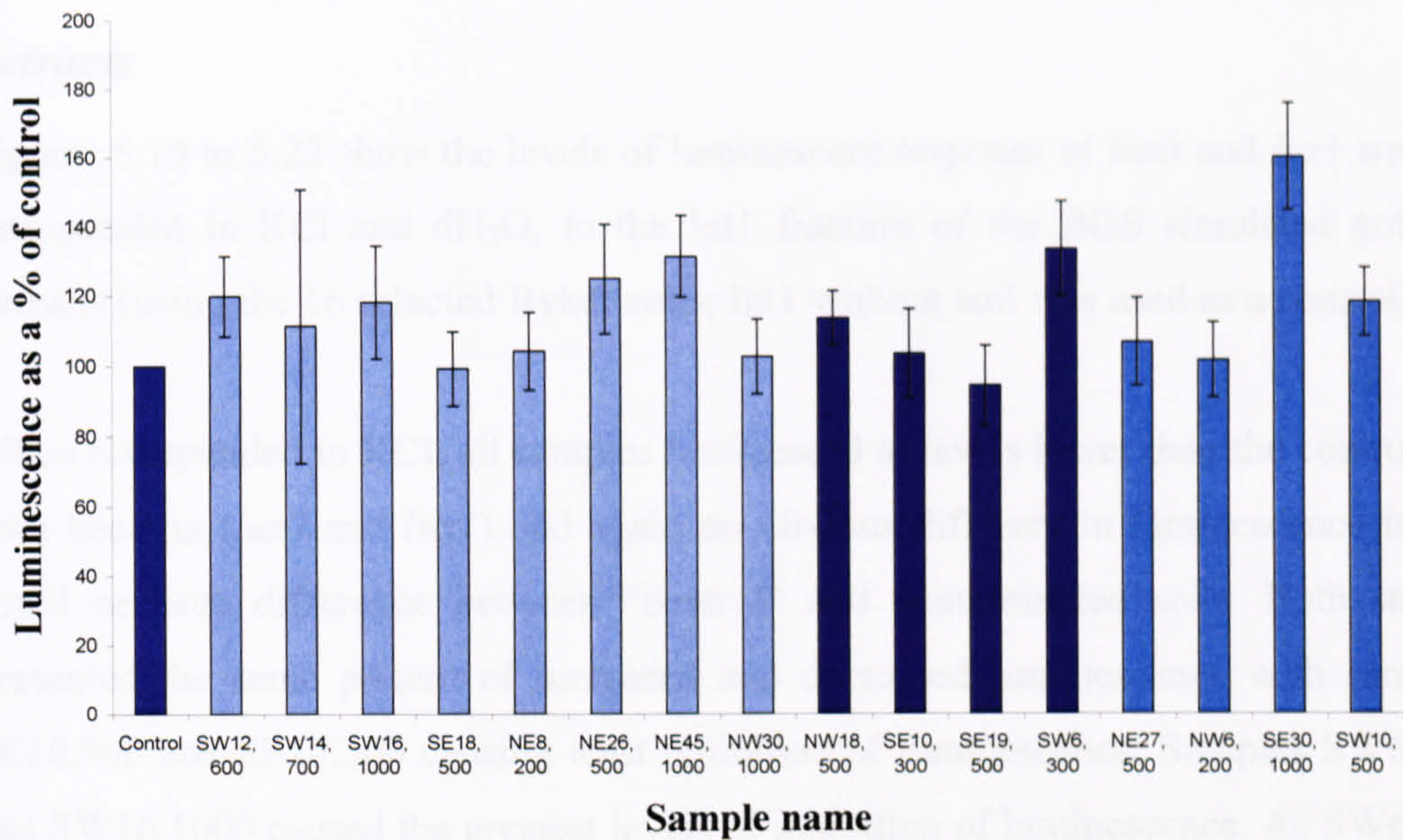


Figure 5.17 Variation in luminescent response of *lux3* exposed to soil/water extracts from urban soil samples. dH_2O used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3.

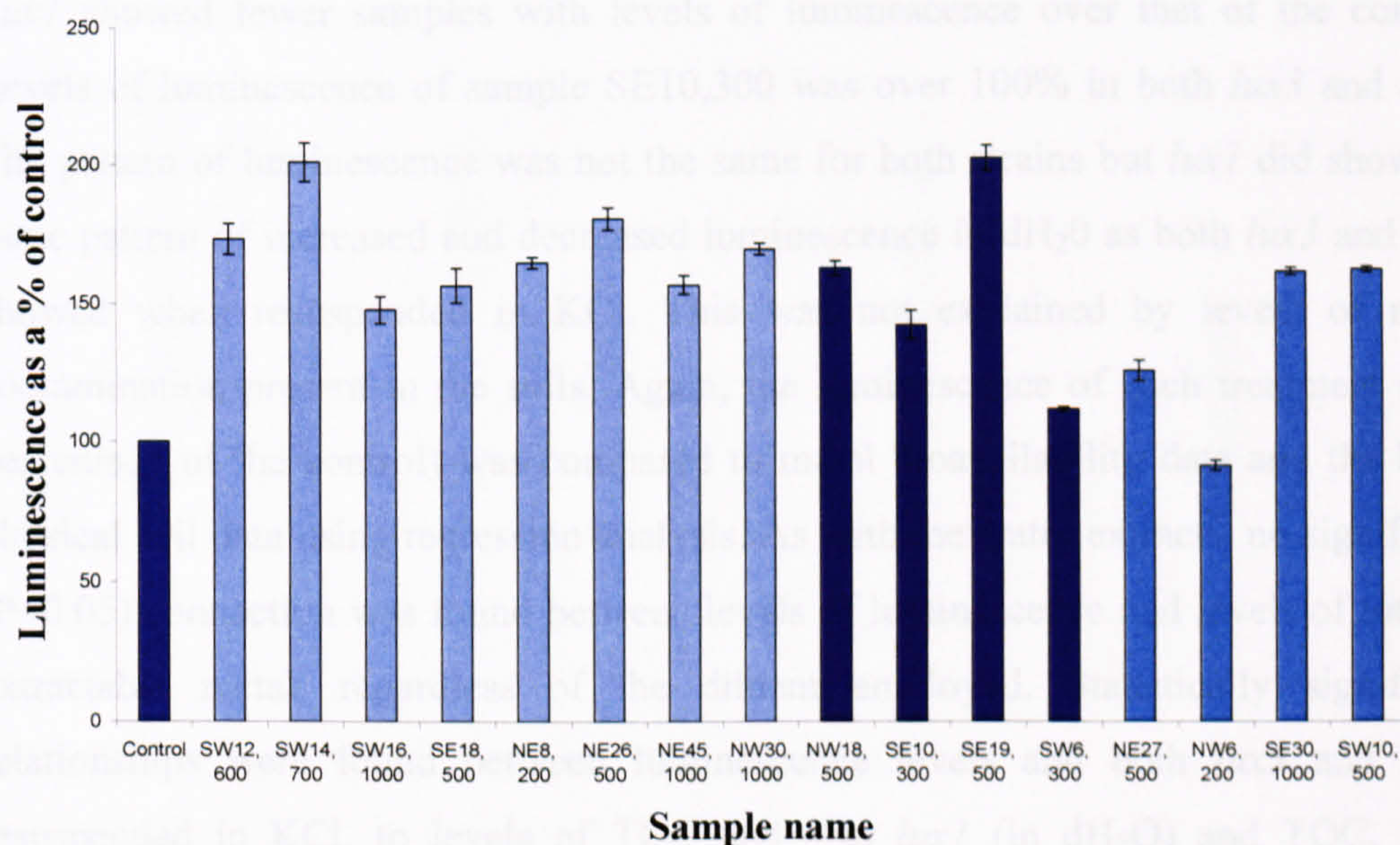


Figure 5.18 Variation in luminescent response of *lux1* exposed to soil/water extracts from urban soil samples. dH_2O used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3.

5.3.4 Sensitivity of luminescent Pseudomonads to soil/simulated gut extracts

Figures 5.19 to 5.22 show the levels of luminescent response of *lux*3 and *lux*1 strains, resuspended in KCl and dH_2O, to the Int1 fraction of the BGS simulated gut/soil extracts (using the 16 selected Byker soils; Int1 without soil was used as a control).

When resuspended in KCl, all samples luminesced at levels lower than the control for both bacteria (*lux3* and *lux1*) and again no obvious different in luminescence levels could be seen difference between "control" and contaminated soils. Both strains presented the same pattern of increased and decreased luminescence with samples SE18,500 and SE10,300 causing least inhibition of luminescence. Samples SW6,300 and SW16,1000 caused the greatest levels of inhibition of luminescence. As SW6,300 has the highest levels of extractable metal, it is possible that the levels of inhibition seen were connected to metal toxicity.

Several samples caused levels of luminescence over the control value (100%) to *Lux3,* resuspended in dH_20. Both "control" and contaminated soils elicited the same effects. *Lux1* showed fewer samples with levels of luminescence over that of the control. Levels of luminescence of sample SE10,300 was over 100% in both *lux3* and *lux1.* The pattern of luminescence was not the same for both strains but *lux1* did show the same pattern of increased and decreased luminescence in dH_20 as both *lux3* and *lux1* showed when resuspended in KCl. This was not explained by levels of metal contamination present in the soils. Again, the luminescence of each treatment (as a percentage of the control) was compared to metal bioavailability data and the basic physical soil data using regression analysis. As with the water extracts, no significant ($P<0.05$) connection was found between levels of luminescence and levels of total or extractable metal, regardless of the diluent employed. Statistically significant relationships were found between luminescence levels and both *lux3* and *lux1,* resuspended in KCl, to levels of TOC and also *lux1* (in dH_2O) and TOC. *Lux3* resuspended in dH_2O was not significantly correlated to TOC. Overall, no relationship was seen between total levels of metal in soil or that extracted into the Int1 fraction of the BGS extract and luminescent response.

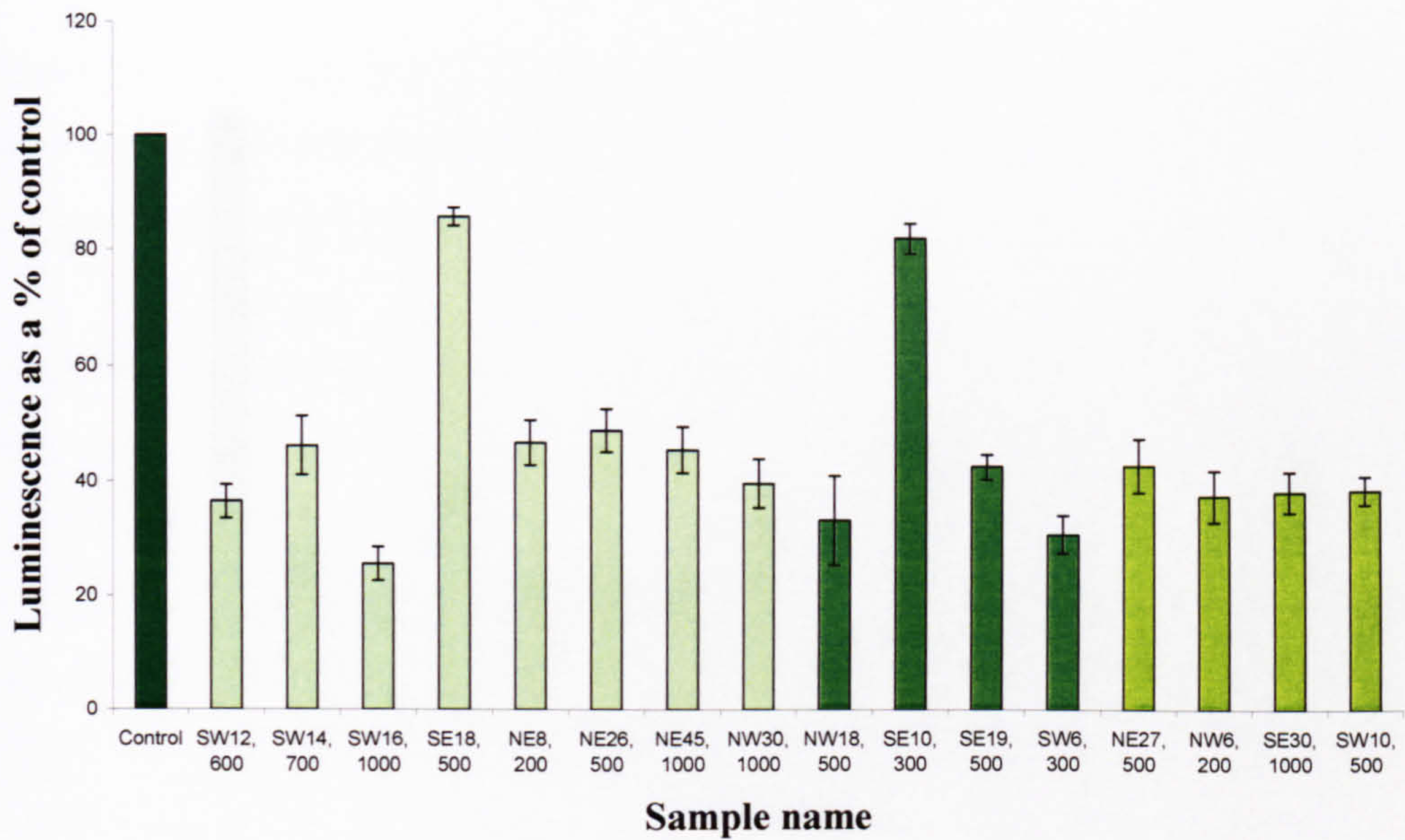


Figure 5.19 Variation in luminescent response of *lux3* exposed to soil/simulated gut extracts from urban soil samples. 0.1 M KCL used as a diluent.

Results are expressed as a percentage of the control +/-SEM n=3

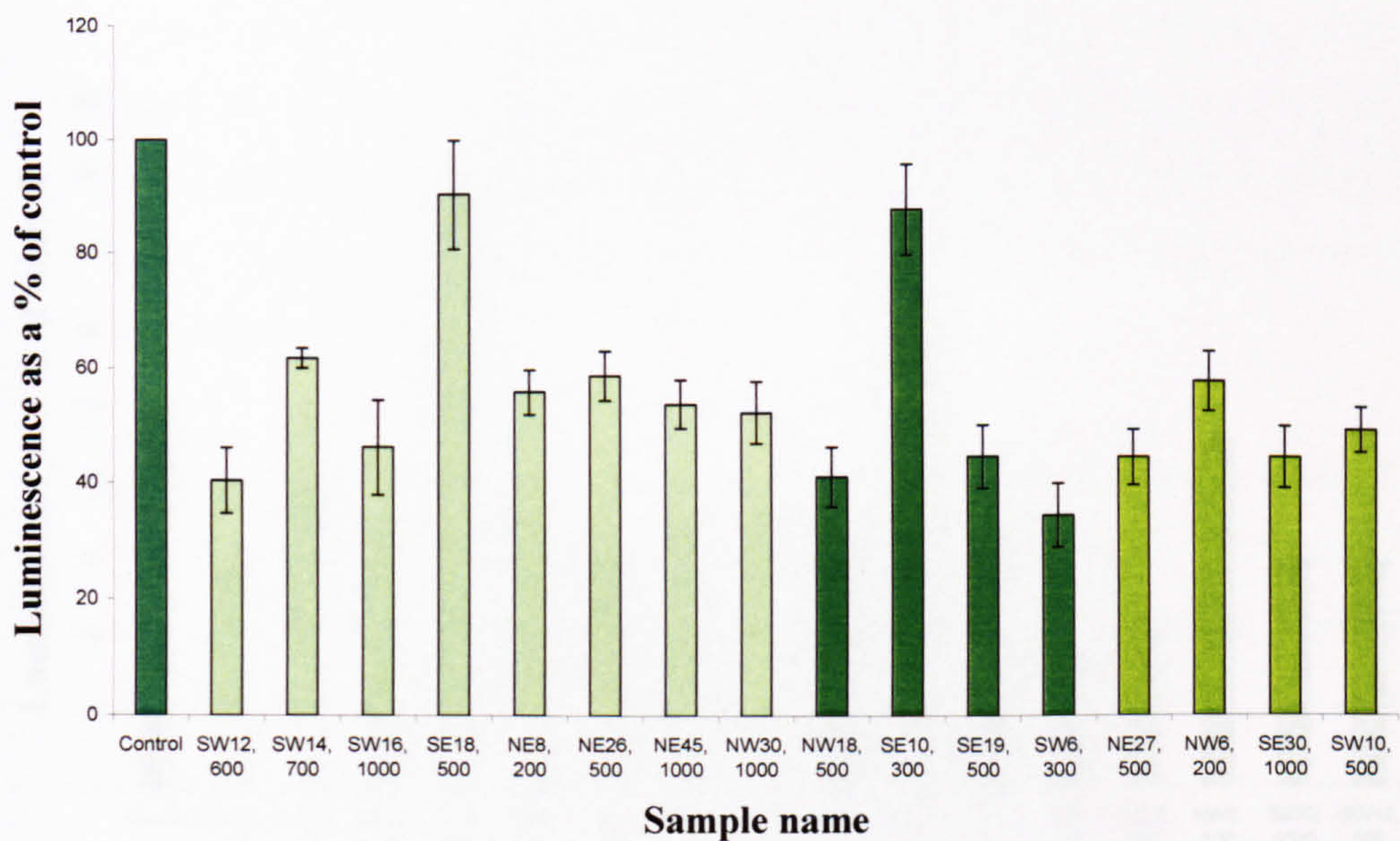


Figure 5.20 Variation in luminescent response of *lux1* exposed to soil/simulated gut extracts from urban soil samples. 0.1 M KCL used as a diluent.

Results are expressed as a percentage of the control +/-SEM n=3

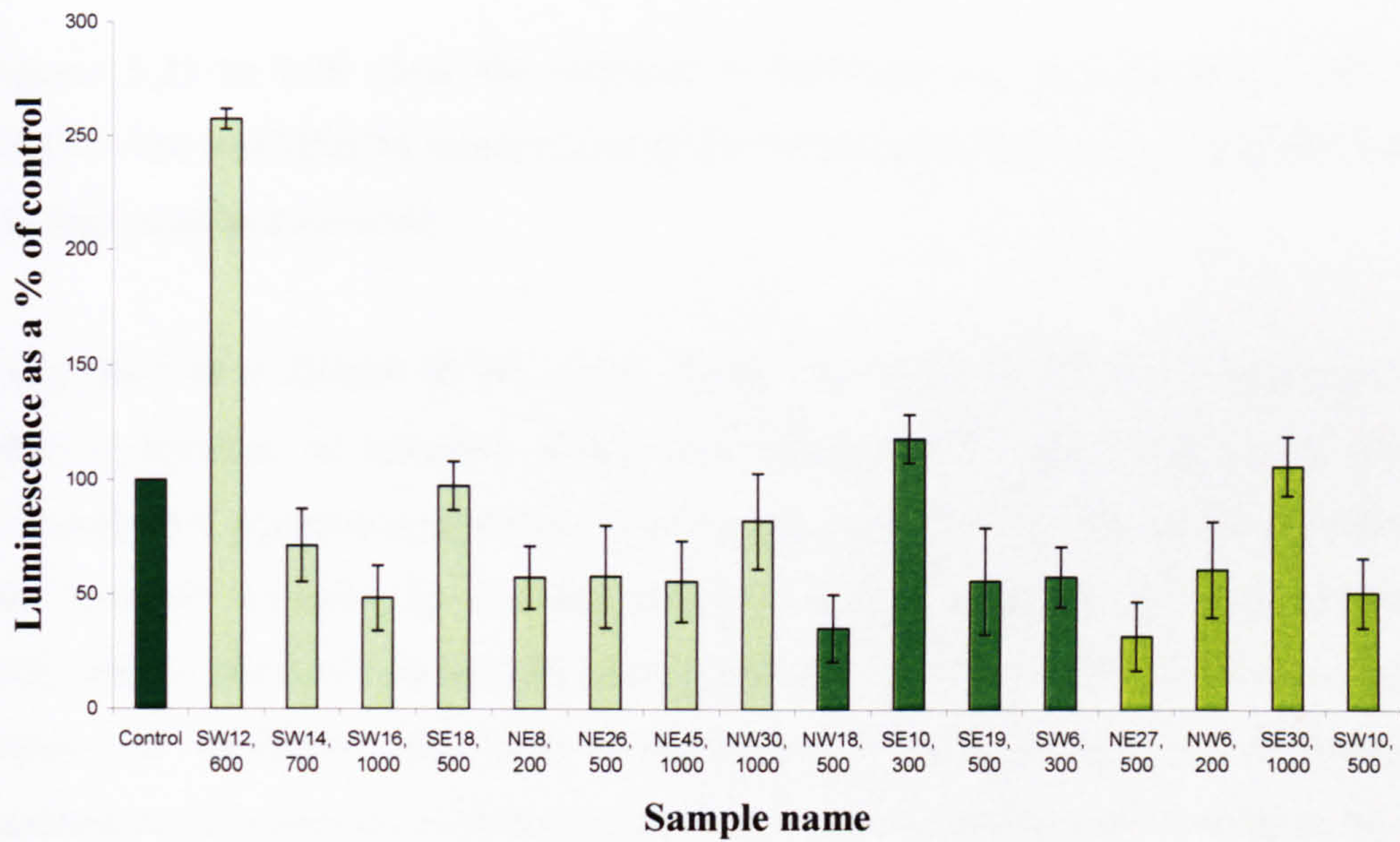


Figure 5.21 Variation in luminescent response of *lux3* exposed to soil/simulated gut extracts from urban soil samples. dH_2O used as a diluent.

Results are expressed as a percentage of the control +/-SEM n=3

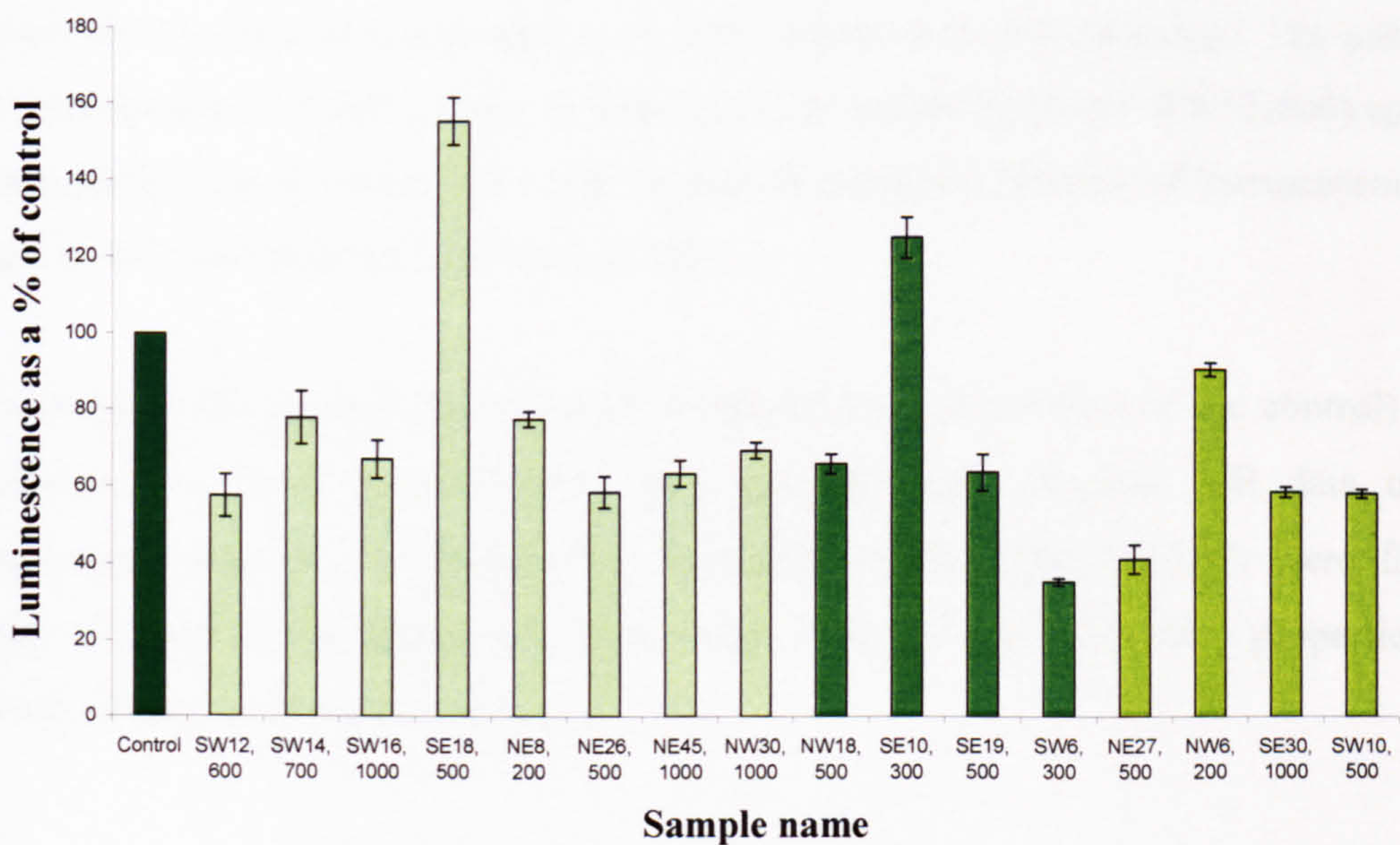


Figure 5.22 Variation in luminescent response of *lux1* exposed to soil/simulated gut extracts from urban soil samples. dH_2O used as a diluent.

Results are expressed as a percentage of the control +/-SEM n=3

5.3.5 Sensitivity of luminescent Pseudomonads to soil/DMEM extracts

Figures 5.23 to 5.26 show the response of *lux*3 and *lux*1 resuspended in KCl and dH_2O to the soil/DMEM extracts (using the 16 selected Byker soils; DMEM without soil was used as a control).

Using KCl as a diluent in the assay, strain *lux3* exhibited levels of luminescence, when exposed to all samples, which were between 80% and 120% of the control. Contaminated samples appeared to show no greater levels of luminescence inhibition that "control" samples. In contrast, levels of luminescence of *lux1,* resuspended in KCl, when exposed to all samples were between 120% and 160% of the control. No pattern of similarity was seen between the 2 bacteria and no difference in luminescence levels was seen due to the total or extractable levels of metal in the soils.

The levels of luminescence of *lux3* seen, when resuspended in dH_20, were all between 100% and 200% of the control (100%) except sample SW12,600. Metal content of the soil appeared to have no influence of luminescence levels. *Lux1* (in dH_20) all showed luminescence levels between 80% and 180% of the control (100%). Total and extractable metal levels appeared to have no influence on luminescence. The patterns of luminescence of both strains, in response to all samples (except SW12,600) appear comparable. Some similarities could be seen between the patterns of luminescence of *lux1* in KCl and both *lux3* and *lux1* in dH_20.

Once again, the luminescence of each treatment (as a percentage of the control) was compared to metal bioavailability data and the basic physical soil data using regression analysis. No statistically significant relationships ($P<0.05$) were found were between luminescence levels of either strain or any of the soil properties or levels of total or extractable metals.

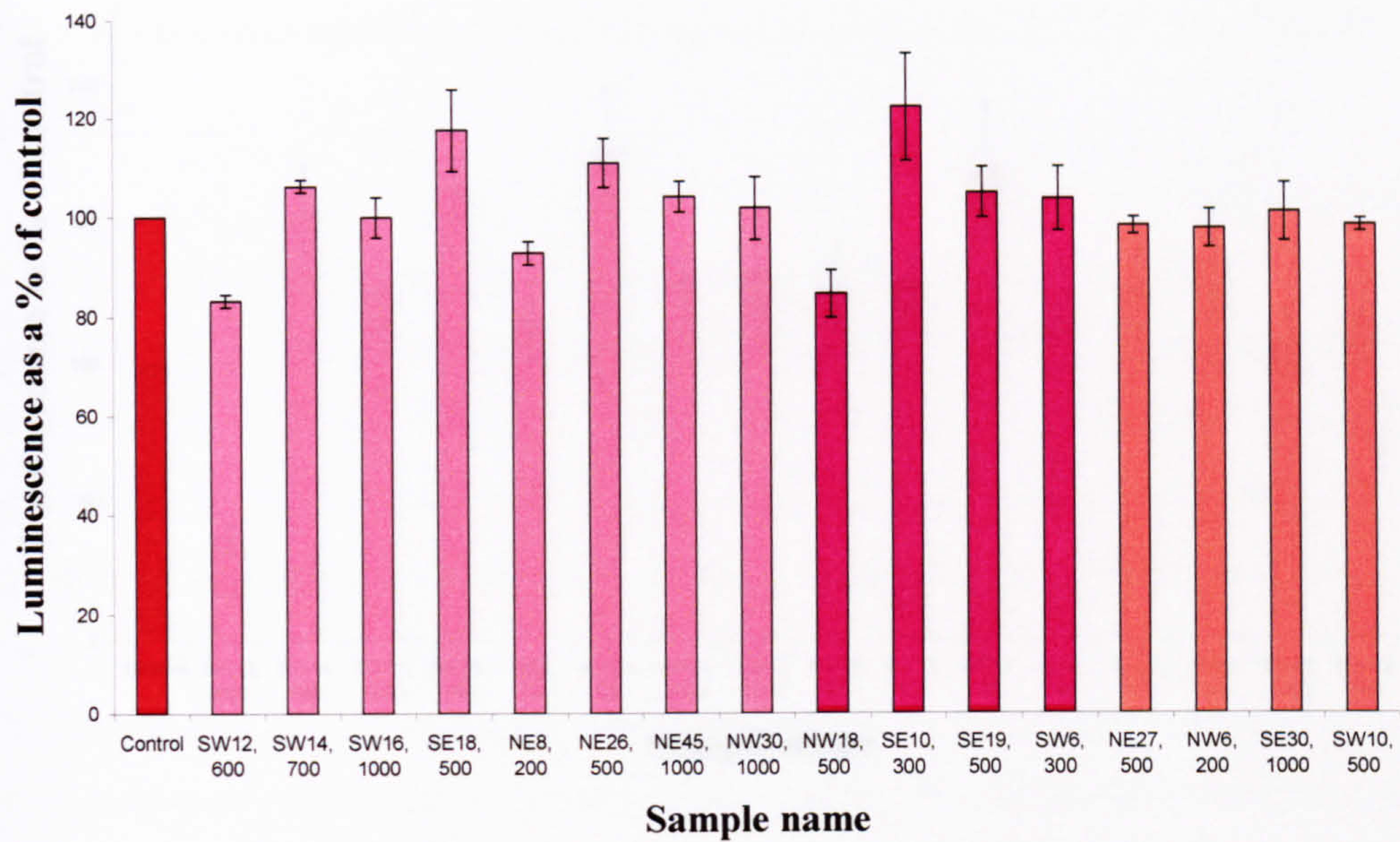


Figure 5.23 Variation in luminescent response of *lux3* exposed to soil/DMEM extracts from urban soil samples. 0.1 M KCL used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3

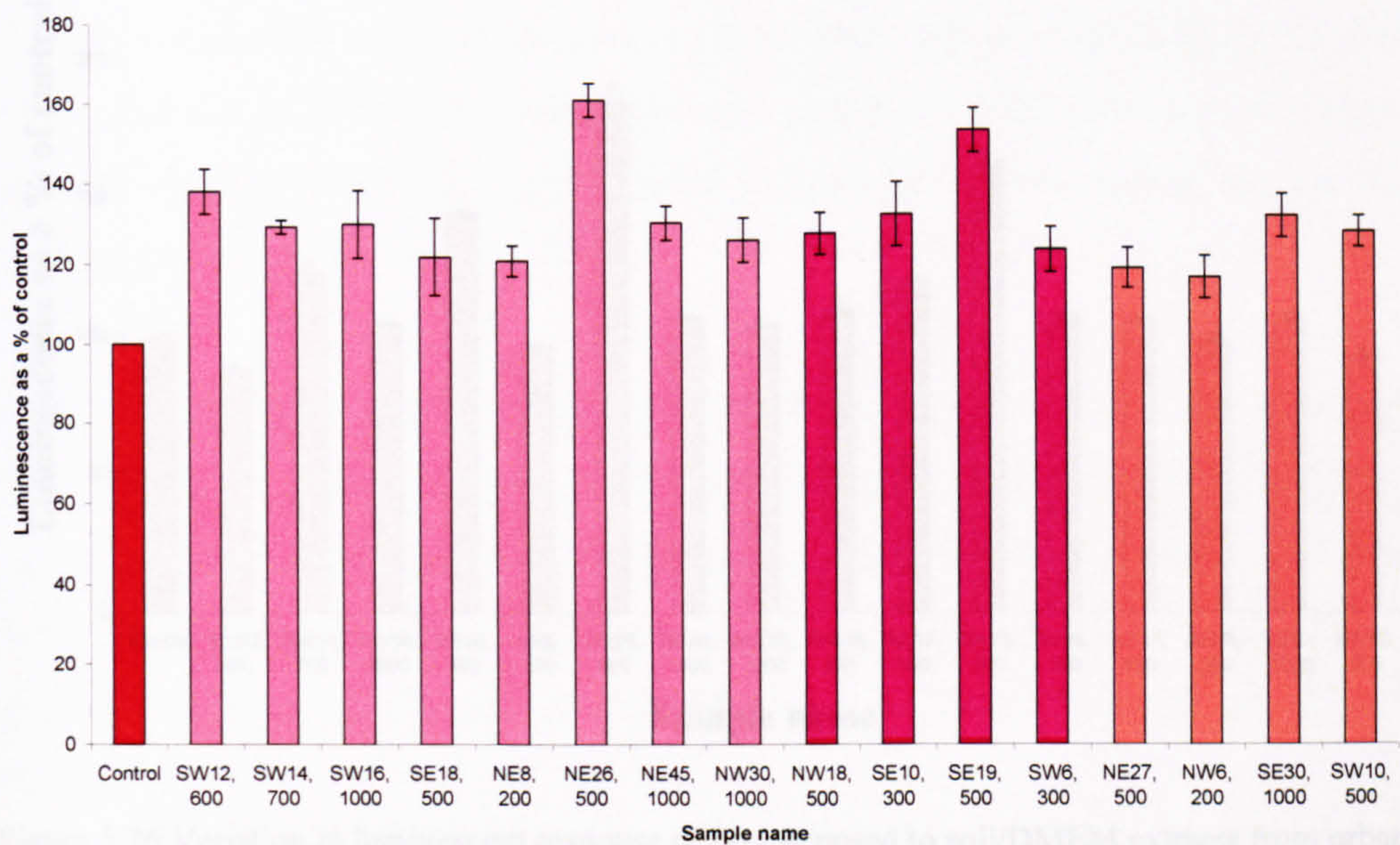


Figure 5.24 Variation in luminescent response of *lux1* exposed to soil/DMEM extracts from urban soil samples. 0.1 M KCL used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3

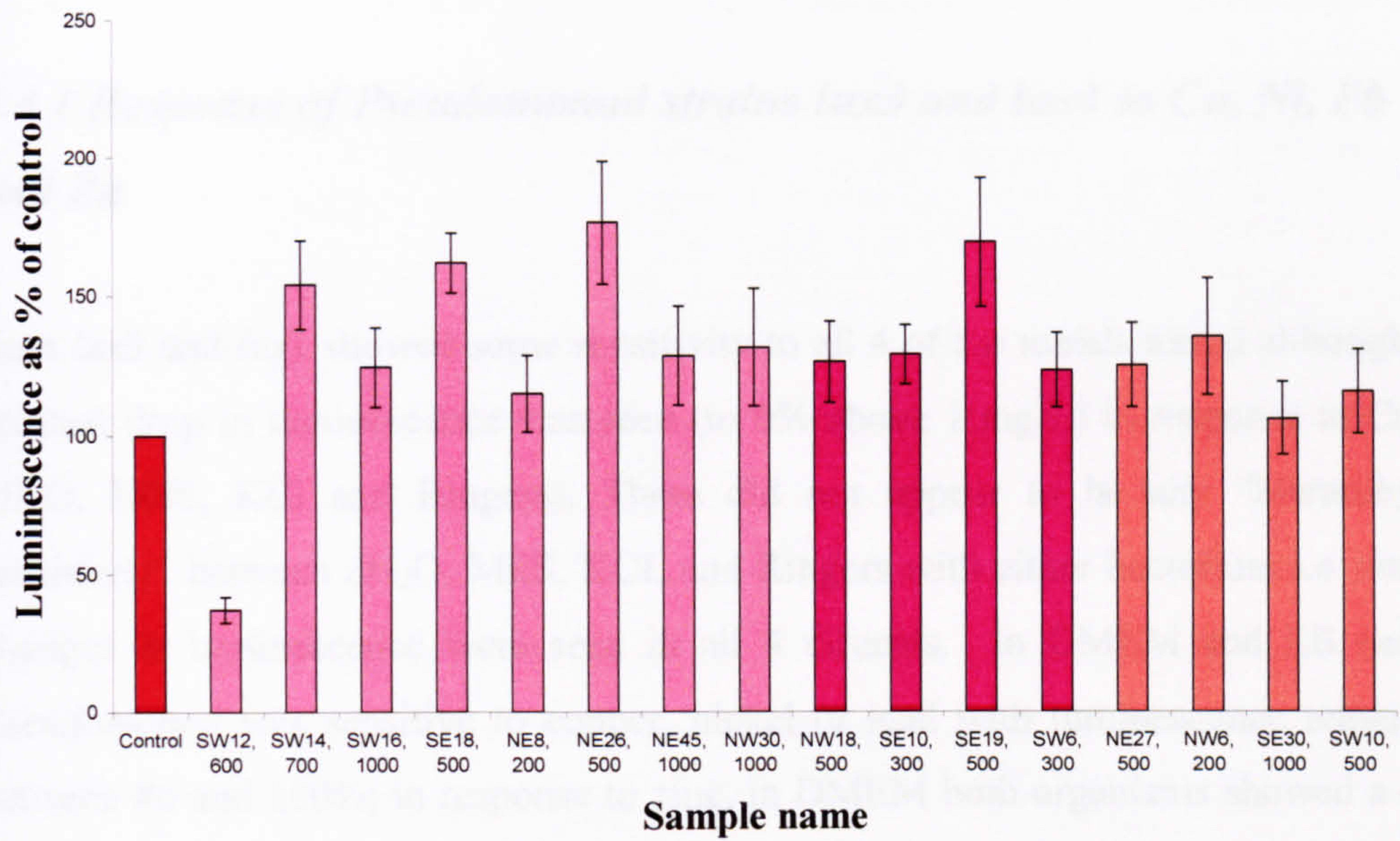


Figure 5.25 Variation in luminescent response of *lux3* exposed to soil/DMEM extracts from urban soil samples. dH_2O used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3

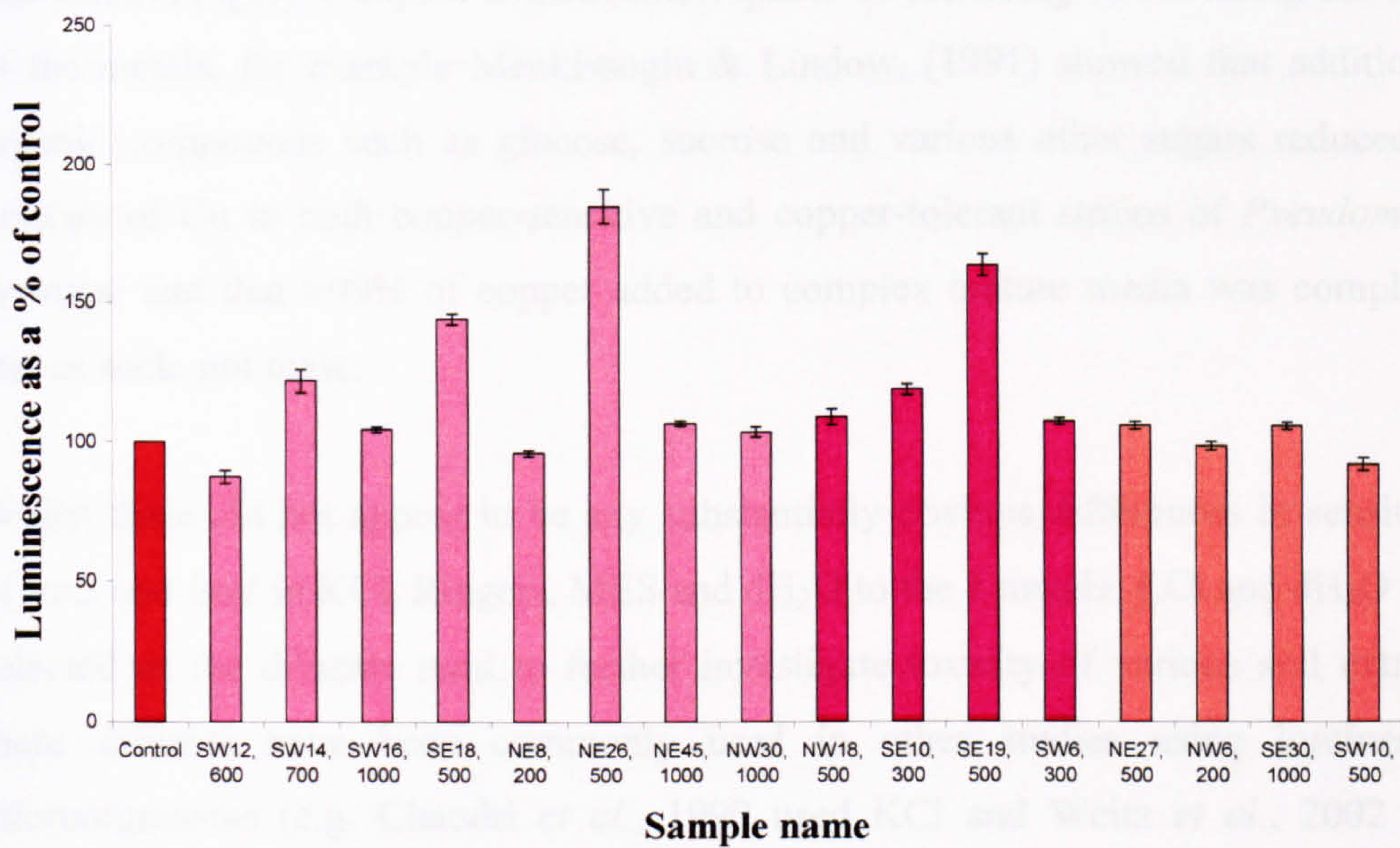


Figure 5.26 Variation in luminescent response of *lux1* exposed to soil/DMEM extracts from urban soil samples. dH_2O used as a diluent. Results are expressed as a percentage of the control +/-SEM n=3

5.4 DISCUSSION

5.4.1 Response of Pseudomonad strains lux3 and lux1 to Cu, Ni, Pb and Zn

Both *lux*3 and *lux*1 showed some sensitivity to all 4 of the metals tested although the greatest drop in luminescence was seen (to 0% above 1 mg/L) in response to Cu (in dH_2O, MES, KCl and Ringers). There did not appear to be any "hierarchy of sensitivity" between dH_2O, MES, KCL and Ringers with either bacterium i.e. similar changes in luminescence were seen in all 4 diluents. In DMEM and LB neither *Pseudomonad* was sensitive to copper, nickel or lead with luminescence remaining between 80 and 100%; in response to zinc, in DMEM both organisms showed a drop in luminescence below 20% above 5 mg/L and in LB, luminescence dropped below 80%.

Both LB and DMEM are cell growth media (bacteria and human cell respectively) and contain highly complex constituents capable of absorbing or buffering the effect of the metals, for example Menkissoglu & Lindow, (1991) showed that addition of organic compounds such as glucose, sucrose and various other sugars reduced the toxicity of Cu to both copper-sensitive and copper-tolerant strains of *Pseudomonas syringae* and that >99% of copper added to complex culture media was complexed and as such, not toxic.

Whilst there did not appear to be any substantially obvious differences in sensitivity of *lux3* and *lux1* in KCl, Ringers, MES and dH_2O to the 4 metals, KCl and dH_2O were selected as the diluents used to further investigate toxicity of various soil extracts; these diluents have been commonly used in other studies using luminescent microorganisms (e.g. Chaudri *et al.*, 1999 used KCl and Weitz *et al.*, 2002 used dH_2O).

Comparing the luminescent response (of both *lux3* and *lux1*) to the 4 metals on a molarity basis would put the hierarchy of toxicity at Cu>Pb>Zn>Ni, see Table 5.2

Table 5.2. Conversion of mg/L of Cu, Ni, Pb and Zn to μM – comparative table.

mg/L	Cu in μM	Ni in μM	Pb in μM	Zn in μM
1	15.7	17.0	4.8	15.3
2	31.4	34.0	9.6	30.6
5	78.6	85.1	24.1	76.4
10	157.3	170.3	48.2	152.9

Other studies that examined the sensitivity of various metals to luminescent micro-organisms found many different results depending on the extracts used, metals (and concentrations) examined and organisms used.

For example Paton *et al.*, (1995) found that a lux modified *Pseudomonas* was more sensitive to Cu and Zn than to Ni, with both chromosomally and plasmid encoded lux genes. Weitz *et al.*, (2002) compared the sensitivity of 2 lux modified *Pseudomonads* to 2 luminescent fungi and found comparable sensitivity to Cu (EC_{50} range between 0.08 and 2.25 mg/L) but the response to zinc was entirely different; the bacteria were both sensitive to Zn ($EC_{50}<0.1$ mg/L) whereas one fungus was moderately sensitive ($EC_{50}<32.1$ mg/L) and the other showed minimal reduction in luminescence ($EC_{50}>90$ mg/L).

Chaudri *et al.*, (1999) assessed Zn and Cu (and Cd) nitrates in mixtures and singly (0-2 mg/L) with an *E.coli* and a *P.fluorescens.* The *Pseudomonas* was found to be more sensitive than the *E.coli* when in combination and increased exposure time significantly increased toxicity. Fulladosa *et al.*, (2005) used the Microtox® assay to establish dose response curves for 10 metals including Zn, Pb and Cu and using EC_{20} values found the toxicity of Pb>Cu>Zn (range 0-2 mg/L).

Overall, toxicity appears to be dependant on the microorganism used although Cu appears to be toxic to all the organisms examined. The *Pseudomonas* stains *lux3* and *lux1* used here appear to be as sensitive to metals as other microbes used in previous work.

5.4.2 Response of lux3 and lux1 to soil extracts

Given the range of sensitivity exhibited by the two *Pseudomonads*, it would be expected that luminescence should decrease most markedly in reaction to samples that had Cu>1 mg/L available i.e. to SE19,500 (BGS and DMEM extracts), SW6,300 (BGS and DMEM extracts), NW6,200 (DMEM extract) and SE30,100 (DMEM extract). A reduction in luminescence may also have been seen with Zn>1 mg/L i.e. NW18, 500 (BGS and DMEM extracts) and SW6, 300 (BGS extract) (see Table 5.1). Statistical analysis showed no indication that the levels of bioavailable (or total) metal in the soils extracts had any influence on luminescence levels of either organism.

The soil/simulated gut extracts showed firm correlation between TOC and luminescence (*lux3* in water being the only exception). DMEM extracts showed no relationship to any of the measured chemical or physical parameters. These results taken together would suggest that an unknown factor had the most influence over luminescence levels. This may be an organic pollutant that was not tested for e.g. PAH, PCB or a pesticide, or another physical property of the soil for examples some organic compound naturally present in the soil. Luminescent response may also be a combination of several factors (probably depending on where the lux gene is in the chromosome). These could include metabolic/growth stimulation from soil nutrients, for example, Hund, (1997) showed that in an algal growth inhibition test, growth was stimulated by nutrients, therefore masking any toxic response) causing an increase in luminescence, this effect would obviously be different from soil to soil and depend on the extract used.

An unexpected increase in luminescence in response to stress could be due to an effect known as "hormesis", a dose-response phenomenon characterized by stimulation at low-dose and inhibition at high dose (Calabrese, 2004). For example Reid *et al.* (1998) saw an increase of luminescence in lux marked biosensors when exposed to PAH solutions when a decrease was expected. This combination of stimulation (increase) and toxicity (decrease) makes interpretation of results difficult.

5.4.3 Summary

In conclusion, investigation into the toxicity of Cu, Ni, Pb and Zn to two luminescent *Pseudomonads* using a range of diluent solutions during the toxicity assay showed that growth media (in this case LB and DMEM) are inappropriate for use. Both bacteria were most sensitive to Cu. Neither organism appeared to be sensitive to known levels of metal in the soil extracts with the only enduring connection between lux response and soils being levels of organic carbon in the soils (when using simulated gut intestinal fraction). This indicates that metal was not available to the organisms probably due to complexation.

It is possible that *lux3* and *lux1* are sensitive to organic contamination (e.g. untested for PAH's or PCB's). Although whilst the levels of dioxins extracted into solution were unknown, the samples with high total levels of dioxins (SW12,600 etc) did not appear to reduce luminescence more than samples with low dioxins.

Problems with the methods used included lack of knowledge as to where the *lux* gene insertion was and therefore what effects were being shown, and "interference" in the luminescent response by differing levels of soil nutrients in each sample.

Regardless of these results, the microbial bioassay approach to testing of contaminated soils has a strong future (given the advantages of cost, time and resource), particularly commercially, as barriers to use in the field are overcome. The relevance of a bacterial response to humans (i.e. the species barrier) will mean that use of microbes as a screening tool and as part of a test battery will most likely continue both experimentally and commercially.

Chapter 6 – General Discussion

6.1 – INTRODUCTION

Contamination of land from both current and historic sources is thought to be widespread in urban areas in the UK (and internationally) but the scale of the problem has yet to be quantified. High levels of contamination causes concern as it may present a risk to human health or vulnerable ecosystems. Both the extent of contamination and also the risk it may pose is presently unknown. Current contaminated land guidance in the UK asks first for a site to be assessed using the source-pathway–receptor model. If there is then found to be contamination and a route to a vulnerable receptor, then further assessment of the risks (possibly leading to remediation) should take place. Forms of further assessment are not currently proscribed. Both the biological availability of contamination to cause harm, and toxicity testing of contaminated soils are areas of research that may complement chemical assessment and provide a more thorough and valid picture of the risks contaminated land may pose.

The aims of this project were:-

- To determine the extent of soil contamination in an urban UK environment.
- To determine the influence of the Byker incinerator on levels of soil contamination.
- To further investigate the risks any contaminated soils may pose to human health using a range of chemical extraction methods that mimic biological extractability.
- To develop a novel and relevant human cell *in vitro* toxicity assay to investigate the toxicity of contaminated soils
- To develop and optimise a bioassay using a native soil organism to further assess the toxicity of contaminated soils

6.2 – SUMMARY AND DISCUSSION

Soil contamination in urban Tyneside around the Byker incinerator

Chemical analysis of 163 soil samples, taken from around the Byker incinerator in urban Tyneside, provided two major investigative findings: firstly that the Byker incinerator was only responsible for increases in levels of contamination of dioxins and metals within the plant boundaries. Secondly, that over half the sites sampled could be deemed to be contaminated using UK contaminated land regulations, i.e. levels of soil contamination were relatively high. Vizard et al. (2003) concluded that the majority of the existing contamination was most probably historical e.g. the Allhusen's alkali works (high levels of dioxin contamination) and the Ouseburn valley lead works. The concerns of local residents about the effects of the incinerator were the stimulus for this project (and other related projects – see Pless-Mulloli e*t al.*, 2000, 2001a, b and 2002) and it is hoped that the results of this study (i.e. Chap. 2 and Vizard *et al.,* 2003) will reassure people of the safety of incinerators used to dispose of domestic waste. The findings of this thesis seem to be fairly typical of various international studies into the effects (or lack of) of incinerators on surrounding soils in urban areas. For example Nouwen *et al.* (2001) investigated the effects of 2 incinerators in an urban part of Belgium, focussing on dioxin levels in soils (again prompted by local resident concern) and found the incinerators were not the main source of dioxins and no meaningful health risk (caused by the incinerator) would ensue from living in the area. The work of Schuhmacher and Domingo and colleagues in Spain into the effects of incinerators has been the most extensive and exhaustive. In one study they found no increase in levels of heavy metals over time (near the incinerator) (Meneses *et al.*, 1999) however around a second incinerator (Nadal *et al.*, 2005), levels of metals changed (some increase, others decrease) but the contribution of the incinerator was minimal compared to other sources. These conclusions have been backed up by further studies by the same research group (Meneses *et al.*, 2004; Nadal *et al.*, 2002). This fear of detrimental health effects and increased contamination from incinerators appears to be a running theme in many communities but not one that is backed up by investigations. The findings of this project are especially pertinent as the UK government Waste Strategy 2006 (www.defra.gov.uk) has outlined how rates of waste disposal through incinerators, (preferably coupled to energy generation) will need to rise from 9% to 25%. As the Byker plant was

originally run as an energy-from-waste unit to service the Byker wall housing area, it is possible this may be utilised again.

Historical urban contamination

The finding that ~55% of sites in this central urban area of Newcastle may be regarded as contaminated (i.e. have levels of contamination over SGV's) also has backing in the literature. As described in section 1.1 (urban contamination), a BGS survey of Sheffield found >60% sites to be over soil guideline values for As.

Extrapolating from this study in Newcastle and the BGS study in Sheffield, it is likely that the legacy of industrialisation in the UK has left over half the urban areas in UK with levels of contamination that are over the current CLEA guideline values. Land contamination is also a global issue, not one confined to the UK, the EU or USA. A wide range of studies investigating levels of metals and dioxins have taken place internationally e.g. Wilcke *et al.*, (1998) study in Bangkok, Thailand and Moller *et al.* (2005) study in Damascus Syria. In all these studies the contamination "source" has been investigated but the "pathway" and "receptor" part of the model need to be further examined to assess any risk. Given that the soil guideline values in the UK (as part of the CLEA model) are based on lengthy exposure, the location of sample sites needs to be taken into account. The majority of soils taken in this study came from public open spaces, the side of roads, middle of parks and verges in industrial estates. Soils taken from the Walker Road allotment site come closest to the "residential with plant uptake" category and users may therefore have a higher risk of exposure to any contaminated soils. Since the samples were taken (in 2002) the site has been fully remediated (see section 2.4.6). Overall, using the source-pathway-receptor model of the 163 sites sampled only a small percentage (9 samples) could potentially be considered contaminated. A limitation of this data set is that the majority of the sites selected for study were in public open spaces (deemed necessary for ease of sampling) and as such unlikely to have a source-pathway-receptor linkage.

However, as the populations of many UK cities expand and planners try to preserve the greenbelts, these contaminated public open spaces may become prime urban development areas and it will be important to keep a record of any future human and ecosystem risk that may occur from these sites. If these sites do pose a risk then it is

clearly impractical to remediate over half of all urban areas (where would all the clean soil come from and contaminated soil go to?) and too costly for any government (national or local) to consider.

Risk assessment

If a pathway and a receptor has been found for specific contaminated sites then the current UK contaminated land guidelines then calls for further assessment to find out if it actually poses any kind of risk. Questions that still need to be answered include:- what is the real cost of contaminated land to human health and what are the implications? How are ecosystems really affected? What risk assessment should be carried out and what tools should be used? In the case of the Walker Rd allotments close to the Byker incinerator (that had received ash from the incinerator onto pathways), once the contamination source and the human receptors were identified, further investigations were carried out into potential exposure pathways. Levels of dioxins and heavy metals were assessed in eggs and vegetables produced at the allotments. A clear link was found between ash use in chicken pens and levels of dioxin contamination in eggs (Pless-Mulloli *et al.*, 2001b). Assessment of vegetables showed that there was either very little (metals) or no (dioxins) evidence of any transfer of contamination from the ash or soil (Pless-Mulloli *et al.*, 2001a). Further risk assessment in this case seemed both necessary and prudent.

Bioaccessibility and bioavailability of contaminants: potential use for risk assessment

Given the high level of historical soil contamination and potential for many of the sites sampled to pose a future risk (due to site development) it was decided to examine selected soil samples to determine their associated risk. As total contamination levels are known to be a poor measure of risk then this work examined different methods to determine the bioaccessibility of metals to humans. Additional studies determined the potential for metals to leach into local waterways (ecosystem risk).

A sub-set of 16 soils were selected from the 163 taken around the Byker incinerator; four with low levels of metals and dioxins, four with low metals and high dioxins, four with medium levels of metals and low dioxins and four with very high levels of

metals and low dioxins. Due to financial constraints and considering the total ranges of amounts of the metals in the soils, Cu, Ni, Pb and Zn were selected for further investigation.

The popularity of "bioavailability" as a concept (and the limitation of "total" levels of contamination) for the further assessment of contaminated soils is evident with the separate evolution of a range of simulated gut bioaccessibility extracts in several countries (see Table 3.1). Assuming that a source-pathway-receptor had been found, this work set out to determine the percentage of soils that presented a risk based on biological extractability. A range of extract methods was used in this study including a water leaching method, 2 simulated gut assays and a cell culture medium leaching method. The results of the water leaching method showed very low levels of all 4 metals, in mg/kg, mg/L and as a percentage of the original "total", were extractable from the soils. These results would indicate that there is a low risk of the metals in these soils leaching away and polluting local water courses (e.g. the river Tyne) and "ecosystems".

Two simulated gut assays were performed; the RIVM and BGS methods. Both assays produced reasonably similar results. Cu was found to have the greatest range of extractability with 2 samples (SW6, 300 and SE19, 500) exceeding the guideline values using the BGS method (only SW6,300 was over guideline using the RIVM method). Ni and Pb extractability was consistently low with both assays. Levels of extractable Zn ranged more than Ni and Pb but less so than Cu; using the RIVM method no samples were over the guideline value whereas using the BGS method, 2 samples (SW6,300 and NW18,500) were over the guideline value. The BGS method was technically easier to carry out (time, cost of materials and complexity) than the RIVM and produced a more comprehensive picture of the human digestive process (i.e. represented various gut compartments; stomach and small intestine) with measurements taken at different stages of the assay.

The cell culture medium (DMEM) leaching extract method produced results that were similar to the BGS and RIVM methods, and was simpler and quicker to carry out. Extractable levels of Cu were found to have a wide range and one sample (SW6, 300) was found to have extractable levels above the guideline value (140mg Cu was found

to be extractable/bioaccessible per kg soil). Levels of extractable Ni and Pb were again very low. Zn again had a greater range than Ni and Pb but no samples were found to have levels over the guideline value. These results were very similar to those of Langley-Turnbaugh *et al.* (2005) who also used cell culture media to examine "biosolubility" of metals from soils (in this case DMEM/F12+CCS was used). Cu was found to be biologically available whereas Ni and Pb (Zn was not investigated) did not appear to be extractable into the biological medium.

The disadvantages of using these extractability techniques include that the extracts still require chemical analysis and then may not be suitable for further use in toxicity testing. Additionally it is still unknown whether the metals extracted using the simulated gut methods would actually be taken up *in vivo*. Chronic effects of exposure are also not addressed using these methods.

As pointed out in the NRC report (National Research Council, 2002) and Ehlers & Luthy, (2003), simplistic extraction techniques such as the water leaching method (and to a certain extent the simulated gut methods) cannot account for the complexity and individual variations of dose, diet uptake or ligand complexation. The conclusion the authors drew (and that the results obtained in this study would probably reinforce) was that extractions tests are best used as screening tools; the report then goes on to recommend further assessment with biological tools (bioassays) as these methods would be able to incorporate factors into toxicity assessment such as transport across membranes and assimilation efficiency of contaminants. The advantages of *in vitro* simulated gut methods are they are both time and cost effective (compared to *in vivo* investigations) and may be very useful to screen large numbers of samples. Limitations included that these methods have only been validated for certain metals, and it is possible that the extract methods may alter metal behaviour so as not to accurately reflect potential toxicity.

***In vitro* assessment of toxicity using a human cell line model**

The need for further work to investigate whether soils officially regarded as contaminated (over SGV's), are actually a threat to human or ecosystem health, is outlined in the CLEA guidelines but particular methods of assessment are not specified. The different soil guideline values that exist internationally are based on a

range of different assumptions (receptor, length of exposure, what constitutes toxicity etc) and there are large gaps in knowledge as to the actual toxic effect of these contaminated soils to humans or ecosystems. *In vitro* test methods, if capable of reflecting *in vivo* conditions and responses to chemicals or soils, could be very useful tools for toxicity assessment. In this thesis it was found that the human liver cell line, HepG2, was sensitive to Cu and Zn at environmentally relevant levels (as found in Chap. 3). Cytotoxic damage was caused to cells by Cu between 10 and 50 mg/L and levels of DNA damage increased significantly from untreated control cells using doses of 0.1 mg Cu/L (and above). Cytotoxic damage was seen in HepG2's dosed with 10 mg/L Zn in the neutral red and glutathione assays and at 50 mg/L with the trypan blue and MTT assays. Levels of DNA damage significantly higher from levels of damage in control cells was observed after exposure to 0.1 mg Zn/L (and above).

The HepG2 cells were exposed to a variety of soil extracts (water, simulated gut and DMEM). A range of problems occurred with method optimisation, more specifically with sterilisation of soil extracts. Cytotoxicity assays were carried out and found that regardless of levels of total metal contamination in soil, the actual soils, or something extracted from the soils, exerted a cytotoxic effect on the HepG2 cells. It was not possible to carry out comet assay work on the HepG2's exposed to soil extracts as the cells were so physically degraded that insufficient DNA remained to measure levels of DNA. It was concluded that the combination of HepG2 cells, soil extracts and the comet assay were not suitable to assess the toxicity of metal contaminated soils.

Several unexpected and interesting findings were made in the course of these investigations. The first was that Zn appeared to be cytotoxic to the HepG2 cells, and at a lower level than Cu. Usually Zn has been found to have a protective effect against cell damage due to other metals (e.g. Cu or Cd) (Fernandez *et al.*, 2003; Singh *et al.*, 2006). Very little academic research to date appears to have focussed on the effects of over-exposure to Zn; this may be due to no (current) knowledge of any genetic conditions which result in inappropriate accumulation of Zn (Wilson's disease is a genetic condition in which Cu is accumulated to toxic levels in humans, see Daniel *et al.*, 2004).

A finding that may merit further research was the significant levels of DNA damage occurring from such low levels (0.1 mg/L of both Cu and Zn) of metal exposure. The comet assay will always show a basic level of DNA breaks due to normal levels of DNA replication (as a precursor to cell replication) occurring in a healthy cell population. An extended dosing regime which allowed time for DNA repair may give more thorough answers as to whether the DNA damage caused by the low levels of metal used are physiologically significant.

The difference in comet tail shape produced by the two metals is also worthy of note. A traditional comet tail shape was seen in cells exposed to Cu whereas exposure to high levels of Zn (10 mg/l and over) a "hedgehog" shape appeared. It is possible that this shape and the high levels of damage seen were due to apoptosis of the cells rather than damage specifically targeted at the DNA. A major flaw of comet assay is that it is only able to pick up recently apoptotic cells; if cells are too damaged they cannot be included in the results (for example see Fig 4.38-4.40), skewing the data by showing lower levels of damage than may actually be occurring.

This study showed HepG2 cells to be sensitive to the metals tested and therefore useful as a screening tool, but one specific cell type cannot be said to be truly representative of all human cell types. Another interesting finding was the discrepancy between the chemical data produced by the cytotoxicity assays (Figures 4.34 to 4.36) and the visual evidence of the phase contrast microscope pictures (Figure 4.33 and Appendix 4) and confocal microscope pictures (Figures 4.38 to 4.40). The cytotoxicity data showed the levels of cell viability when exposed to irradiated DMEM (no soil) to be the same as cells exposed to non-irradiated DMEM (no soil). The pictures of the cells actually show extensive changes in cell morphology and debris in the surrounding media suggesting major damage effects to the cell population.

Assessment of toxicity using native soil organisms

Although the main focus of most contaminated land guidance is human health, potential risks to ecosystems should also be examined (see section 1.4, Ecological risk assessments and Environment Agency, 2003). Use of luminescent microorganisms (particularly native soil organisms that have had a "lux" gene engineered into the

genome) to assess the toxicity of many different chemicals and compounds and also the effects of contaminated land is increasing in popularity (both experimentally and commercially) due to economic and methodological considerations. In this study, two strains (*lux3* and *lux1*) of luminescent *Pseudomonas* (that had not been previously optimised for use with metals) were investigated in respect of sensitivity to Cu, Ni, Pb and Zn (0-10 mg/L range). Optimisation of methods included trialling both bacteria with 6 diluent solutions (KCl, Ringers, dH_2O, DMEM, MES and LB). DMEM and LB were found to be unsuitable for use in toxicity assays as luminescent response to increasing metal was not affected, probably due to metal complexation by constituents of the two growth media. Both *lux3* and *lux1* appeared to be more sensitive to Cu with levels of luminescence dropping to 0% of control at higher metal levels. When exposed to Ni, Pb and Zn levels of luminescence never dropped to 0% at any tested concentration. KCl and dH_2O were selected to further investigate the toxicity of extract solutions (see Chp3) of selected Byker soils. Soil/water extracts showed no connection between levels of total or extractable metal and reduction in luminescence (as a % of control). Interestingly, levels of luminescence (nearly) always stayed below the level of the control when resuspended in KCl whereas when resuspended in dH_2O, luminescence of samples was generally greater than control. Statistical analysis showed no connection between levels of luminescence and soil pH, particle size of TOC.

Exposure to the soil/simulated gut extracts (using the "Int1" fraction of the BGS method) again showed no relationship between levels of luminescence and total or extractable metal. Statistical analysis showed that luminescence levels of both *lux3* and *lux1* in KCl and *lux1* in dH_20 were significantly related to levels of TOC. Exposure to the soil/DMEM extracts again showed no connection between levels of luminescence and total or extractable levels of metal and also no connection to the physical properties (pH, particle size, TOC) of the soils.

Whilst there were few statistically significant connections between levels of luminescence and the measured soils properties, some factor in each sample was responsible for either stimulating or repressing levels of luminescence. Some samples showed drops in luminescence to 30% of the control whilst rises were up to 200% of the control. Further thought needs to be given to whether these changes in

luminescence have any greater implications; what factor or combination of factors is causing these changes? Does a drop in luminescence mean that a soil ecosystem is damaged by the contamination?

6.3 -CONCLUSION

In conclusion, the key findings of this thesis were that the Byker incinerator did not appreciably contribute to levels of dioxin or heavy metal contamination in urban Tyneside, other than within the plant boundaries. The high levels of contamination in existence are thought to be historical. Due to changes in government waste policy, it is important that incinerators are found not to increase health risks to the general public. However this study has shown contamination of soils by dioxins and heavy metals in the urban Tyneside area to be widespread. Assuming a source-pathway-receptor, further assessment of a subset of samples showed that very low levels of metal were extractable (bioaccessible) from the soils and that total levels of metal are not a good predictor of extractability. Of the 16 samples investigated, 8 had total levels of metal above SGV's. Only three samples were found to have bioaccessible level of metal (Cu and Zn) above the SGV's. Results were found to be reproducible with different methods (simulated gut, DMEM) used.

A human cell line (HepG2) was shown to be sensitive to Cu and Zn at environmentally relevant levels. A range of cytotoxicity assays showed Zn to reduce cell viability at a lower concentration that Cu. Both metals caused a significant ($P<0.05$) increase in levels of DNA damage compared to control cells, over a 24 hr exposure period. Unfortunately, the HepG2 cells were not found to be suitable as a toxicity assay for metal contaminated soils due to problems harmonising cell culturing techniques and both the physical nature of soil and the high levels of microfauna. Native soil organisms (two strains of a luminescent *Pseudomonas*) were also shown to be sensitive to metals (Cu, Ni, Pb and Zn) at environmentally relevant levels in a range of diluents. Exposure to soil extract solutions did not show any link between total or extractable levels of metals and levels of luminescence.

6.4 – PROVISIONAL FUTURE WORK

Studies that could be undertaken in the future to further investigate the results generated in this study include:-

- Analysis of the speciation of metals in the Byker soils in order to see whether this would be a better predictor of bioavailability (and hence potential toxicity).
- Extended dosing of HepG2 cells with Cu and Zn to investigate whether DNA damage seen at very low dose levels is repaired over time.
- Examine the sensitivity of human cells to mixtures of metals, particularly Cu and Zn (looking for the protective effect).
- Trialling human cell lines other than HepG2's that may be "hardier", for example colon or intestinal cell lines.
- Assess the extractability and potential toxicity of the dioxin contaminated soils using a luminescent mammalian cell assay e.g. www.biodetectionsystems.com
- Investigate the potential of luminescent yeast based assays for assessing the toxicity of contaminated soils e.g. www.gentronix.co.uk

Appendix 1

Summary of various international soil guideline values

DIOXINS

Table A1.1 A summary of international soil guideline values and trigger levels for dioxins

Guideline source	**Land-use**	**Dioxins in ng I-TEQ/ kg soil**
CLEA, UK (2002)	No limit set	N/A
ICRCL, UK (1987) NB – withdrawn in 2002	No limit set	N/A
Dutch list (1993)	No limit set	N/A
Dutch list (2001) PCDD/F and PCB	Integrated serious risk concentration (proposed) Current intervention level	360 1000
German soil protection regulations (1999)	Playgrounds Residential areas Parks and recreational areas Industrial and commercial (NB no level set for allotments)	100 1000 1000 10000
USEPA	Remediation goal – residential areas	1000
New Zealand	Remediation goal – residential areas	1500

NB - adapted and revised from Pless-Mulloli *et al.* (2002)

Table A1.2 Summary of international guideline values for levels of As in soils.

Guideline source	Land-use	mg/kg
CLEA, UK (2002) **SGV**	Residential with or without plant uptake and allotments	20
	Commercial and industrial	500
ICRCL, UK (1987) NB – withdrawn in 2002 **Assessment trigger level**	Allotment or garden	10
	Sports field	40
	Park	40
Dutch list, Netherlands (1993)	Playground	20-25
	Allotment or garden	20-40
	Sports field	35
	Agriculture	40
Dutch list, Netherlands (2001)	Current intervention level	55
	Integrated serious risk concentration (proposed)	85
German soil protection regulations (1999) **Assessment trigger level**	Playgrounds	25
	Residential areas	50
	Parks and playing fields	125
	Commercial and industrial	140

NB - adapted and revised from Pless-Mulloli *et al.* (2002)

Table A1.3 Summary of international guideline values for levels of Cd in soils.

<table>
<tr><th rowspan="2">Guideline source</th><th rowspan="2">Land-use</th><th colspan="3">mg/kg</th></tr>
<tr><th>pH6</th><th>pH7</th><th>pH8</th></tr>
<tr><td rowspan="4">CLEA, UK (2002)
SGV</td><td>Residential with plant uptake</td><td>1</td><td>2</td><td>8</td></tr>
<tr><td>Allotments</td><td>1</td><td>2</td><td>8</td></tr>
<tr><td>Residential without plant uptake</td><td></td><td></td><td>30</td></tr>
<tr><td>Commercial and industrial</td><td></td><td></td><td>1400</td></tr>
<tr><td rowspan="3">ICRCL, UK (1987)
NB – withdrawn in 2002
Assessment trigger level</td><td>Allotment or garden</td><td colspan="3">3</td></tr>
<tr><td>Sports field</td><td colspan="3">15</td></tr>
<tr><td>Park</td><td colspan="3">15</td></tr>
<tr><td rowspan="5">Dutch list, Netherlands (1993)</td><td>Playground</td><td colspan="3">2-10</td></tr>
<tr><td>Allotment or garden</td><td colspan="3">1-2</td></tr>
<tr><td>Sports field</td><td colspan="3">2</td></tr>
<tr><td>Park</td><td colspan="3">4</td></tr>
<tr><td>Agriculture</td><td colspan="3">2</td></tr>
<tr><td rowspan="2">Dutch list, Netherlands (2001)</td><td>Current intervention level</td><td colspan="3">12</td></tr>
<tr><td>Integrated serious risk concentration (proposed)</td><td colspan="3">13</td></tr>
<tr><td rowspan="6">German soil protection regulations (1999)
Assessment trigger level</td><td>Playgrounds</td><td colspan="3">10</td></tr>
<tr><td>Allotments and gardens used for vegetables and by children</td><td colspan="3">2</td></tr>
<tr><td>Residential areas</td><td colspan="3">20</td></tr>
<tr><td>Parks and playing fields</td><td colspan="3">50</td></tr>
<tr><td>Commercial and industrial</td><td colspan="3">60</td></tr>
<tr><td>Grazing land – Intervention level</td><td colspan="3">20</td></tr>
</table>

NB - adapted and revised from Pless-Mulloli *et al.* (2002)

Table A1.4 Summary of international guideline values for levels of Cr in soils.

<table>
<tr><th>Guideline source</th><th>Land-use</th><th colspan="2">mg/kg</th></tr>
<tr><td rowspan="4">CLEA, UK (2002)
SGV</td><td>Residential with plant uptake</td><td colspan="2">130</td></tr>
<tr><td>Allotments</td><td colspan="2">200</td></tr>
<tr><td>Residential without plant uptake</td><td colspan="2">130</td></tr>
<tr><td>Commercial and industrial</td><td colspan="2">5000</td></tr>
<tr><td rowspan="3">ICRCL, UK (1987)
NB – withdrawn in 2002
Assessment trigger level</td><td>Allotment or garden</td><td colspan="2">600</td></tr>
<tr><td>Sports field</td><td colspan="2">1000</td></tr>
<tr><td>Park</td><td colspan="2">1000</td></tr>
<tr><td rowspan="5">Dutch list, Netherlands
(1993)</td><td>Playground</td><td colspan="2">50-200</td></tr>
<tr><td>Allotment or garden</td><td colspan="2">70-100</td></tr>
<tr><td>Sports field</td><td colspan="2">150</td></tr>
<tr><td>Park</td><td colspan="2">150</td></tr>
<tr><td>Agriculture</td><td colspan="2">200</td></tr>
<tr><td rowspan="3">Dutch list, Netherlands
(2001)</td><td>Current intervention level</td><td colspan="2">380</td></tr>
<tr><td rowspan="2">Integrated serious risk
concentration (proposed)</td><td>Cr III</td><td>Cr VI</td></tr>
<tr><td>220</td><td>78</td></tr>
<tr><td rowspan="3">German soil protection
regulations (1999)
Assessment trigger level</td><td>Playgrounds</td><td colspan="2">200</td></tr>
<tr><td>Residential areas</td><td colspan="2">400</td></tr>
<tr><td>Parks and playing fields</td><td colspan="2">1000</td></tr>
</table>

NB - adapted and revised from Pless-Mulloli *et al.* (2002)

Table A1.5 Summary of international SGV's for levels of Cu in soils.

Guideline source	Land-use	mg/kg
CLEA, UK (2002)	No level set	N/A
ICRCL, UK (1987) NB – withdrawn in 2002 **Assessment trigger level**	Allotment or garden	130
Dutch list, Netherlands (1993)	Playground Allotment or garden Sports field Park Agriculture	50 50 100 200 50
Dutch list, Netherlands (2001)	Current intervention level Integrated serious risk concentration (proposed)	190 96
German soil protection regulations (1999)	No level set	N/A

NB adapted & revised from Pless-Mulloli *et al.* (2002)

MERCURY

Table A1.6 Summary of international SGV's for levels of Hg in soils.

<table>
<tr><th>Guideline source</th><th>Land-use</th><th colspan="2">mg/kg</th></tr>
<tr><td rowspan="4">CLEA, UK (2002)
SGV (Inorganic)</td><td>Residential with plant uptake</td><td colspan="2">8</td></tr>
<tr><td>Allotments</td><td colspan="2">8</td></tr>
<tr><td>Residential without plant uptake</td><td colspan="2">15</td></tr>
<tr><td>Commercial and industrial</td><td colspan="2">480</td></tr>
<tr><td rowspan="3">ICRCL, UK (1987)
NB – withdrawn in 2002
Assessment trigger level</td><td>Allotment or garden</td><td colspan="2">2</td></tr>
<tr><td>Sports field</td><td colspan="2">20</td></tr>
<tr><td>Park</td><td colspan="2">20</td></tr>
<tr><td rowspan="5">Dutch list, Netherlands
(1993)</td><td>Playground</td><td colspan="2">0.5-10</td></tr>
<tr><td>Allotment or garden</td><td colspan="2">2</td></tr>
<tr><td>Sports field</td><td colspan="2">0.5</td></tr>
<tr><td>Park</td><td colspan="2">5</td></tr>
<tr><td>Agriculture</td><td colspan="2">10</td></tr>
<tr><td rowspan="2">Dutch list, Netherlands
(2001)</td><td>Current intervention level</td><td colspan="2">10</td></tr>
<tr><td>Integrated serious risk concentration (proposed)</td><td>Inorganic
36</td><td>Organic
4</td></tr>
<tr><td rowspan="4">German soil protection regulations (1999)
Assessment trigger level</td><td>Playgrounds</td><td colspan="2">10</td></tr>
<tr><td>Residential areas</td><td colspan="2">20</td></tr>
<tr><td>Parks and playing fields</td><td colspan="2">50</td></tr>
<tr><td>Industrial and commercial</td><td colspan="2">80</td></tr>
</table>

NB adapted and revised from Pless-Mulloli *et al.* (2002)

NICKEL

Table A1.7 Summary of international SGV's for levels of Ni in soils.

Guideline source	Land-use	mg/kg
CLEA, UK (2002) SGV	Residential with plant uptake	50
	Residential without plant uptake	75
	Commercial and industrial	5000
ICRCL, UK (1987) NB – withdrawn in 2002 **Assessment trigger level**	Allotment or garden	70
Dutch list, Netherlands (1993)	Playground	40-70
	Allotment or garden	70-80
	Sports field	100
	Park	100
	Agriculture	100
Dutch list, Netherlands (2001)	Current intervention level	210
	Integrated serious risk concentration (proposed)	100
German soil protection regulations (1999) Assessment trigger level	Playgrounds	70
	Residential areas	140
	Parks and playing fields	350
	Industrial and commercial	900

NB - adapted and revised from Pless-Mulloli *et al.* (2002)

Table A1.8 Summary of international SGV's levels of Pb in soils.

Guideline source	Land-use	mg/kg
CLEA, UK (2002) **SGV**	Residential with or without plant uptake	450
	Allotments	450
	Commercial and industrial	750
ICRCL, UK (1987) NB – withdrawn in 2002 **Assessment trigger level**	Allotment or garden	500
	Sports field	2000
	Park	2000
Dutch list, Netherlands (1993)	Playground	200
	Allotment or garden	200-300
	Sports field	200
	Agriculture and parks	500
Dutch list, Netherlands (2001)	Current intervention level	530
	Integrated serious risk concentration (proposed)	580
German soil protection regulations (1999) **Assessment trigger level**	Playgrounds	200
	Residential areas	400
	Parks and playing fields	1000
	Industrial and commercial	2000

NB - adapted and revised from Pless-Mulloli *et al.* (2002)

Table A1.9 Summary of international SGV's for levels of Zn in soils.

Guideline source	Land-use	mg/kg
CLEA, UK (2002) SGV	No level currently set	N/A
ICRCL, UK (1987) NB – withdrawn in 2002 **Assessment trigger level**	Allotment or garden	300
Dutch list, Netherlands (1993)	Playground	300
	Allotment or garden	300
	Sports field	300
	Park	1000
	Agriculture	300
Dutch list, Netherlands (2001)	Current intervention level	720
	Integrated serious risk concentration (proposed)	350
German soil protection regulations (1999) Assessment trigger level	No levels set	N/A

NB - adapted and revised from Pless-Mulloli *et al.* (2002)

Appendix 2

Arsenic and heavy metals

	Metals in mg/kg							
	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
SAMPLE								
SE1, 50	14.41	-0.58	61.36	174.32	0.22	32.72	242.65	816.28
SE2, 100	10.60	-0.75	52.69	111.21	0.25	23.66	198.53	631.55
SE3, 100	9.77	-1.07	23.10	43.47	0.14	10.51	104.49	135.45
SE4, 150	13.56	-0.95	43.94	69.75	0.32	24.65	230.60	347.28
SE5, 150	12.83	0.75	67.21	494.86	0.63	49.04	759.12	546.80
SE6, 200	8.74	-0.57	42.85	32.70	0.17	18.33	83.13	147.95
SE7, 200	37.00	1.78	55.07	189.57	0.97	36.63	723.45	815.47
SE8, 250	30.72	-0.06	62.70	183.80	0.68	37.81	754.67	654.66
SE9, 250	16.29	-0.77	43.39	71.80	0.44	25.02	396.93	373.68
SE10, 300	7.83	1.85	205.24	3377.30	1.53	93.67	2137.39	1418.76
SE11, 300	32.62	0.07	54.94	205.55	0.72	46.68	944.56	707.93
SE12, 350	19.26	-0.25	58.47	134.76	0.41	36.08	2851.48	404.97
SE13, 350	15.54	-0.96	48.39	77.72	0.39	19.47	203.96	236.28
SE14, 400	9.59	-1.05	54.65	71.70	0.30	26.30	152.86	195.45
SE15, 400	21.02	0.31	61.21	195.29	0.94	28.40	552.14	509.61
SE16, 450	10.33	-0.98	58.75	68.95	0.34	29.62	165.13	212.88
SE17, 450	23.34	-1.11	62.55	120.87	0.43	31.13	393.51	288.46
SE18, 500	15.40	-1.35	61.94	40.11	0.06	29.09	49.27	107.48
SE19, 500	43.17	-0.45	86.42	1128.56	0.71	50.59	1287.40	816.99
SE20, 550	10.39	-1.62	68.59	68.64	0.21	26.34	163.19	264.49
SE21, 550	14.63	-1.08	229.77	83.85	0.37	43.28	279.69	277.27
SE22, 600	5.02	-1.64	74.74	19.98	0.03	23.00	40.28	98.77
SE23, 600	20.04	-0.99	79.45	133.06	0.46	35.72	562.31	507.90
SE24, 650	76.25	-1.19	50.95	30.97	0.17	15.85	135.23	85.17
SE25, 650	22.18	-1.01	66.25	130.15	0.27	33.43	848.30	787.19
SE26, 700	7.36	-1.36	73.36	54.18	0.81	31.37	163.06	176.62
SE27, 700	16.85	-1.00	67.77	101.44	0.27	26.91	399.75	401.30
SE28, 750	5.91	-1.94	48.09	25.56	0.10	14.14	46.64	74.60
SE29, 750	41.71	-1.51	73.42	150.12	0.56	46.43	272.87	383.80
SE30, 1000	12.84	-0.88	71.24	1045.76	0.14	22.86	161.14	305.58
SE31, 1000	7.76	-1.08	76.45	46.25	0.16	26.62	115.33	146.18
SE32, 1250	11.92	-1.40	76.40	60.98	0.14	27.18	156.63	176.45
SE33, 1250	16.20	-0.65	70.20	90.31	0.26	30.21	382.51	491.15
SE34, 1500	13.16	-0.87	66.42	122.59	0.32	32.51	292.01	321.86
SE35, 1500	32.42	-0.63	53.85	147.58	0.41	37.99	286.62	500.13
SE36, 1750	29.76	-0.10	54.42	254.92	0.54	37.61	432.89	470.00
SE37, 1750	25.04	-1.82	56.86	66.16	0.44	23.15	546.21	299.28
SE38, 2000	10.50	-1.55	60.58	53.20	0.25	19.49	260.70	266.59
SE39, 2000	8.48	-1.29	54.49	51.95	0.12	20.98	105.99	180.75
SE40, 2250	38.97	-1.59	59.05	161.07	0.59	33.86	326.17	276.83
SE41, 2250	11.48	-1.54	74.93	282.68	0.57	31.08	218.02	474.84

	Metals in mg/kg						
SAMPLE	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
NW1, 50	11.56	1.46	67.61	108.11	0.21	27.05	247.86	505.30
NW2, 100	6.56	-1.57	45.73	38.18	0.11	15.09	92.33	169.79
NW3, 100	31.12	1.30	59.83	208.17	0.57	54.41	329.83	840.58
NW4, 150	57.33	3.84	46.99	376.45	0.63	52.10	568.10	2444.43
NW5, 150	7.97	-0.08	53.69	76.09	0.17	28.84	147.21	326.07
NW6, 200	48.97	0.26	60.00	531.84	0.46	82.27	377.48	470.08
NW7, 200	9.64	-0.29	42.15	40.93	0.17	15.32	163.02	166.00
NW8, 250	43.98	0.78	75.06	543.06	0.75	91.58	561.13	1397.15
NW9, 250	10.31	-0.19	42.42	49.00	0.20	15.74	155.45	164.78
NW10, 300	13.04	-0.26	53.59	60.60	0.22	23.21	151.89	255.21
NW11, 300	9.27	-0.35	36.49	33.00	0.14	15.67	93.70	180.62
NW12, 350	120.47	-0.04	98.57	199.83	0.93	42.36	255.01	438.52
NW13, 350	10.86	-0.11	42.35	54.22	0.14	20.38	131.94	228.25
NW14, 400	24.61	-0.01	35.49	208.51	0.95	39.24	325.69	273.39
NW15, 400	16.77	-0.22	50.29	82.91	0.24	26.41	153.09	287.20
NW16, 450	11.58	-0.23	45.01	50.43	0.17	20.62	180.56	204.68
NW17, 450	16.33	0.41	61.22	139.16	0.21	19.54	599.89	651.97
NW18, 500	59.85	1.38	78.73	284.60	1.98	89.94	918.12	1212.78
NW19, 500	4.75	-0.24	31.85	57.15	0.10	14.69	123.93	341.35
NW20, 550	10.11	-0.30	39.79	53.23	0.16	14.45	136.77	297.24
NW21, 550	29.10	4.83	112.39	364.48	0.60	91.51	1706.19	1394.03
NW22, 600	17.02	0.87	51.81	81.96	4.01	35.72	642.56	381.66
NW23, 600	11.19	0.45	56.23	51.95	0.20	28.24	176.81	305.68
NW24, 650	12.22	0.12	51.91	75.10	0.17	24.41	206.87	332.97
NW25, 650	7.30	-0.02	43.47	32.78	0.24	16.17	70.73	173.07
NW26, 700	6.86	-0.33	42.95	30.99	0.11	39.42	67.05	144.47
NW27, 700	14.14	-0.07	41.99	53.84	0.23	20.09	388.15	400.01
NW28, 750	11.39	-0.15	45.79	52.09	0.26	17.95	140.05	181.33
NW29, 750	7.90	-0.22	38.06	55.36	0.12	18.85	69.73	151.72
NW30, 1000	14.61	0.09	53.14	80.38	0.23	20.25	203.97	267.46
NW31, 1000	15.06	0.20	47.91	68.43	0.64	24.82	284.46	243.64
NW32, 1250	11.30	0.54	50.61	90.87	0.27	19.69	360.97	271.47
NW33, 1250	18.63	0.05	48.76	66.52	0.32	23.37	227.50	473.21
NW34, 1500	16.09	0.02	50.75	64.80	0.43	22.22	199.06	222.61
NW35, 1500	9.80	0.13	55.12	42.57	0.18	17.81	125.95	187.34
NW36, 1750	15.04	0.54	57.68	206.52	0.51	43.52	525.37	772.41
NW37, 1750	12.03	0.51	41.65	48.03	0.27	16.22	191.02	185.70
NW38, 2000	11.88	0.09	45.19	72.21	0.40	22.04	269.48	285.04
NW39, 2000	6.92	-0.04	25.59	54.86	0.56	15.18	236.54	219.80
NW40, 2250	16.08	0.64	56.59	84.12	0.98	35.70	548.95	549.52
NW41, 2250	12.56	0.04	51.88	60.98	0.29	17.51	419.50	266.65

SAMPLE	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
			Metals in mg/kg					
SW1, 50	18.22	0.21	76.99	147.39	0.26	34.75	351.07	1218.62
SW2, 100	36.51	0.07	69.94	241.45	0.48	62.00	318.83	835.88
SW3, 150	15.19	0.02	70.39	177.25	0.31	26.75	211.04	685.70
SW6, 300	278.93	1.97	127.65	1204.69	4.99	71.71	1056.49	1511.31
SW7, 350	39.65	2.03	134.18	2204.19	1.49	70.09	1953.13	1880.25
SW8, 350	19.14	-0.02	31.65	62.78	0.32	21.09	212.00	210.24
SW9, 450	55.55	6.95	124.74	12107.15	3.05	164.53	4134.34	4625.32
SW10, 500	34.49	1.87	68.05	143.81	0.29	26.44	296.39	462.56
SW11, 550	24.04	0.24	85.92	94.50	0.36	45.48	646.54	272.40
SW12, 600	22.73	0.07	54.05	52.61	0.38	27.97	251.93	198.18
SW13, 650	11.68	-0.10	36.87	27.31	0.17	16.06	134.79	130.46
SW14, 700	16.54	0.12	45.50	73.55	0.89	23.57	269.37	235.10
SW15, 750	10.28	0.30	31.97	112.10	3.90	26.44	273.29	232.01
SW16, 1000	29.35	0.07	64.77	88.24	0.51	36.11	266.12	292.60
SW17, 1250	21.76	4.18	41.68	1179.38	1.33	27.07	299.67	2740.02
SW18, 1500	24.65	0.08	52.95	140.75	0.40	34.19	224.87	273.63
SW19, 1750	9.31	-0.20	35.76	34.98	0.28	15.08	135.05	151.04
SW20, 2000	11.24	-0.02	60.39	45.95	0.31	27.24	156.82	211.84
SW21, 2250	21.71	0.53	46.13	98.74	0.83	28.90	494.16	246.59

SAMPLE	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
			Metals in mg/kg					
NE1, 50	9.27	3.14	62.99	85.10	0.38	25.66	343.50	601.21
NE2, 100	5.22	0.36	33.42	29.44	0.12	11.10	77.48	154.71
NE3, 100	10.15	1.41	40.92	68.57	0.32	25.12	487.45	379.81
NE4, 100	6.00	0.44	30.64	47.49	0.29	16.24	359.19	254.07
NE5, 150	7.22	0.35	38.61	71.90	0.23	23.51	323.77	326.42
NE6, 150	6.83	0.31	42.34	75.11	0.23	24.21	361.51	334.50
NE7, 150	9.29	-0.09	48.57	62.28	0.15	17.23	122.60	198.51
NE8, 200	13.67	0.02	41.16	79.96	0.35	21.88	167.76	232.11
NE9, 200	11.75	-0.44	52.56	46.46	0.22	27.21	122.82	202.65
NE10, 200	13.91	-0.08	57.97	85.13	0.37	22.60	211.46	255.78
NE11, 250	13.26	-0.40	49.10	53.93	0.21	21.79	128.80	244.58
NE12, 250	21.55	0.22	51.05	116.20	0.36	31.65	334.02	331.07
NE13, 250	7.88	-0.41	38.39	29.51	0.53	18.43	65.11	131.78
NE14, 300	31.94	-0.04	44.92	124.71	0.57	26.02	384.15	367.72
NE15, 300	13.66	-0.18	54.47	77.57	0.29	29.13	522.38	375.59
NE16, 300	24.85	0.49	51.46	125.44	0.58	34.07	393.96	338.66
NE17, 350	15.31	-0.36	43.66	51.06	0.27	17.27	121.73	191.46
NE18, 350	12.22	-0.14	71.98	110.27	0.83	23.82	196.51	748.53
NE19, 350	19.56	-0.11	31.63	66.27	0.39	21.13	244.77	201.37
NE20, 400	13.94	-0.03	37.94	107.05	0.29	19.93	202.51	368.76
NE21, 400	28.20	-0.05	54.45	103.97	1.53	33.40	299.18	282.20
NE22, 400	15.82	-0.13	51.22	74.24	0.22	23.18	162.64	276.23

SAMPLE	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
				Metals in mg/kg				
NE23, 450	20.03	-0.25	49.53	103.95	0.41	25.40	232.94	256.26
NE24, 450	32.14	-0.06	50.57	106.81	0.71	30.06	372.47	260.57
NE25, 450	6.65	-0.24	31.89	39.36	0.22	17.64	86.21	169.55
NE26, 500	8.40	-0.32	35.24	37.28	0.26	12.68	96.70	130.02
NE27, 500	25.01	-0.04	52.64	135.23	0.67	26.05	251.05	342.43
NE28, 500	8.07	-0.06	49.26	46.08	0.33	17.24	118.32	203.74
NE29, 550	27.93	0.08	43.45	211.03	1.02	28.18	304.61	379.51
NE30, 550	10.32	-0.41	48.14	46.99	0.27	18.20	124.23	171.07
NE31, 550	6.32	-0.37	42.33	42.21	0.22	18.36	94.53	154.29
NE32, 600	11.86	-0.30	46.02	64.18	0.62	25.95	143.35	227.96
NE33, 600	18.57	0.26	46.11	114.42	0.42	25.92	279.24	365.74
NE34, 600	15.54	0.12	40.42	81.01	0.46	24.84	285.69	300.34
NE35, 650	20.42	0.25	44.30	108.00	0.86	33.70	345.77	379.02
NE36, 650	22.47	3.74	51.93	206.18	0.59	38.30	1021.12	862.67
NE37, 650	6.68	-0.22	32.80	29.91	0.12	15.54	59.79	138.01
NE38, 700	17.78	-0.01	48.91	76.82	0.66	28.57	251.59	259.08
NE39, 700	21.79	-0.19	37.96	52.75	0.39	17.97	129.78	165.42
NE40, 700	24.31	-0.03	40.46	76.55	0.66	25.15	218.75	214.14
NE41, 750	33.99	-0.13	51.18	81.67	0.89	22.19	207.12	225.55
NE42, 750	24.47	0.23	51.66	73.44	0.40	31.72	236.00	244.64
NE43, 750	9.23	-0.07	37.95	38.55	0.23	16.39	107.29	200.29
NE44, 1000	23.29	0.27	44.82	92.83	0.43	33.31	269.09	414.27
NE45, 1000	17.41	-0.10	38.53	79.78	0.27	21.66	126.25	192.23
NE46, 1000	15.69	-0.20	57.33	74.81	0.37	29.94	203.99	183.81
NE47, 1250	13.80	-0.21	34.95	50.35	0.25	15.09	140.56	151.38
NE48, 1250	22.86	0.08	41.88	87.01	0.50	25.43	253.30	245.61
NE49, 1250	14.05	0.25	25.97	86.00	0.42	27.22	201.32	295.55
NE50, 1500	29.10	0.28	60.16	107.70	0.43	35.77	435.88	443.12
NE51, 1500	7.14	-0.22	43.44	32.26	0.15	15.38	88.18	112.93
NE52, 1500	16.66	0.17	42.13	110.15	0.36	31.42	260.65	392.09
NE53, 1750	7.84	0.27	44.41	42.69	0.12	15.68	118.17	255.81
NE54, 1750	10.39	-0.08	30.99	42.02	0.18	13.58	80.40	129.37
NE55, 1750	11.11	0.21	43.19	51.48	0.27	37.90	114.68	356.78
NE56, 2000	15.65	-0.09	36.78	56.00	0.25	16.66	115.91	153.57
NE57, 2000	12.17	0.32	65.70	67.43	0.31	20.12	137.79	167.03
NE58, 2000	17.84	-0.34	39.37	56.10	0.28	23.47	150.07	170.76
NE59, 2250	14.67	0.09	51.32	97.83	0.77	32.89	339.08	407.29
NE60, 2250	20.32	0.01	37.04	78.19	0.30	20.92	286.12	412.54
NE61, 2250	17.65	-0.28	47.00	41.62	0.18	18.00	78.28	128.16

Sample	Total PCDD/F in ng I-TEQ/kg	PCDD/F Pattern	Sample	Total PCDD/F in ng I-TEQ/kg	PCDD/F Pattern
SE1, 50	36.36	I	NE1, 50	553.73	I
SE2, 100	31.38	D	NE2, 100	34.00	I
SE3, 100	18.33	I	NE3, 100	71.84	I
SE6, 200	11.76	I	NE4, 100	90.11	I
SE7, 200	55.85	D	NE8, 200	18.90	D+(I)
SE10, 300	18.75	O	NE9, 200	15.90	I
SE11, 300	51.64	D	NE10, 200	33.90	I
SE14, 400	27.00	O	NE14, 300	37.89	D+(I)
SE15, 400	38.90	D	NE15, 300	24.38	Chemical
SE18, 500	481.37	F	NE16, 300	62.14	D+(I)
SE19, 500	48.76	O	NE20, 400	26.05	D
SE22, 600	6.86	D	NE21, 400	43.41	O
SE23, 600	32.62	D	NE22, 400	24.64	I
SE26, 700	79.62	D+F	NE26, 500	9.06	D+(I)
SE27, 700	37.51	D	NE27, 500	24.53	D+(I)
SE30, 1000	154.00	F+D+C	NE28, 500	14.52	D+(I)
SE31, 1000	17.33	D	NE32, 600	25.24	D
SE34, 1500	80.17	D+(F)	NE33, 600	56.68	D
SE35, 1500	71.99	D+(F)	NE34, 600	26.65	D+(I)
SE38, 2000	39.72	D+(F)	NE38, 700	22.03	D+(I)
SE39, 2000	16.08	D	NE39, 700	15.37	C
			NE40, 700	22.59	D
NW1, 50	494.47	I	NE44, 1000	34.00	O
NW2, 100	30.00	I	NE45, 1000	13.34	D+(I)
NW3, 100	121.00	I	NE46, 1000	16.00	D
NW6, 200	66.14	I+D	NE50, 1500	38.00	OCDD
NW7, 200	11.68	D+I	NE51, 1500	5.98	D
NW10, 300	25.94	OCDD	NE52, 1500	23.26	D
NW11, 300	6.58	OCDD	NE56, 2000	26.15	O
NW14, 400	94.77	OCDD	NE57, 2000	27.70	D+(O)
NW15, 400	18.57	OCDD+ D	NE58, 2000	36.64	D+(O)
NW18, 500	102.21	D			
NW19, 500	9.55	OCDD	SW1, 50	83.53	I
NW22, 600	36.53	D+OCDD	SW2, 100	53.68	D+I
NW23, 600	13.34	OCDD+D	SW6, 300	38.69	I+OCDD
NW26, 700	8.45	OCDD+D	SW10, 500	435.22	F1+F2
NW27, 700	21.15	D+(I)	SW12, 600	1771.58	F1+F2
NW30, 1000	16.23	D	SW14, 700	1910.99	F1
NW31, 1000	15.87	D	SW16, 1000	1173.89	F2
NW34, 1500	10.92	D	SW18, 1500	69.36	F+O+C
NW35, 1500	9.75	D	SW20, 2000	14.00	D
NW38, 2000	12.00	D			
NW39, 2000	15.36	D			

NB PCDD/PCDFF patterns I=incinerator, D=deposition, F=furan, and OCDD= OCDD. For further explanation see Vizard et al (2003).

APPENDIX 3

Additional data from Chapter 3

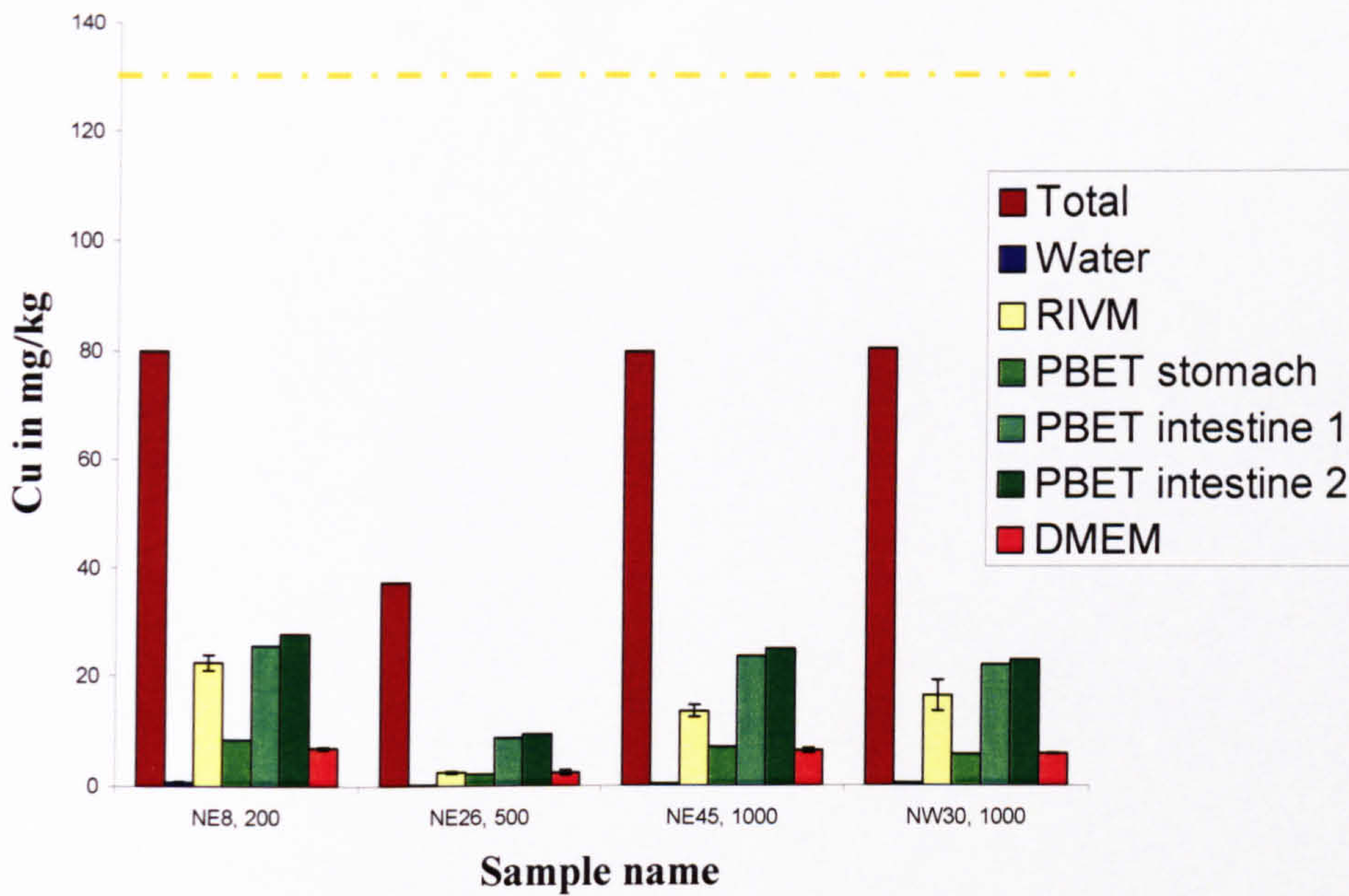


Figure A3.1 - Levels of Cu in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed yellow line indicates guideline value for Cu – 130 mg/kg.

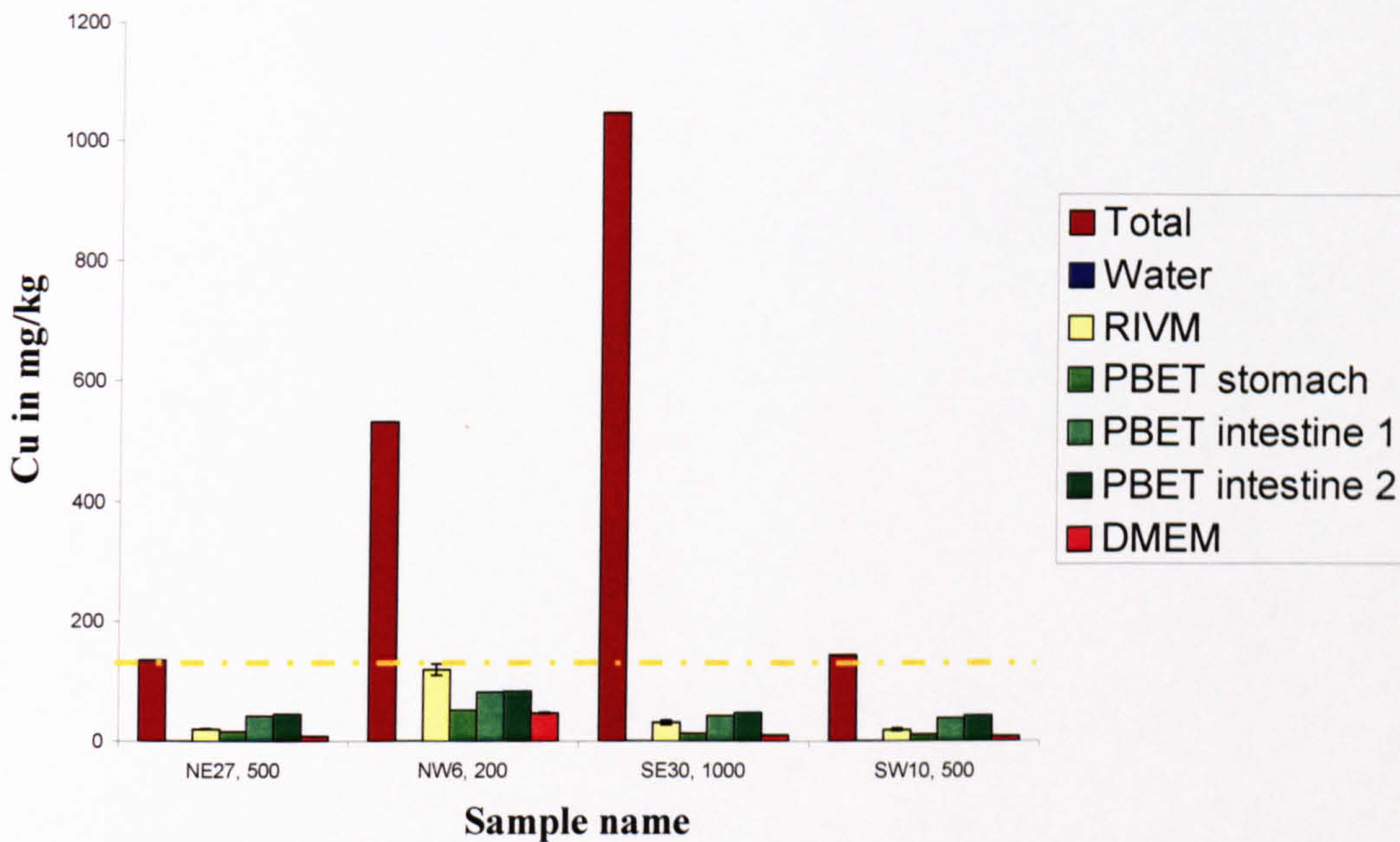


Figure A3.2 - Levels of Cu in 4 heavily contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed yellow line indicates guideline value for Cu – 130 mg/kg.

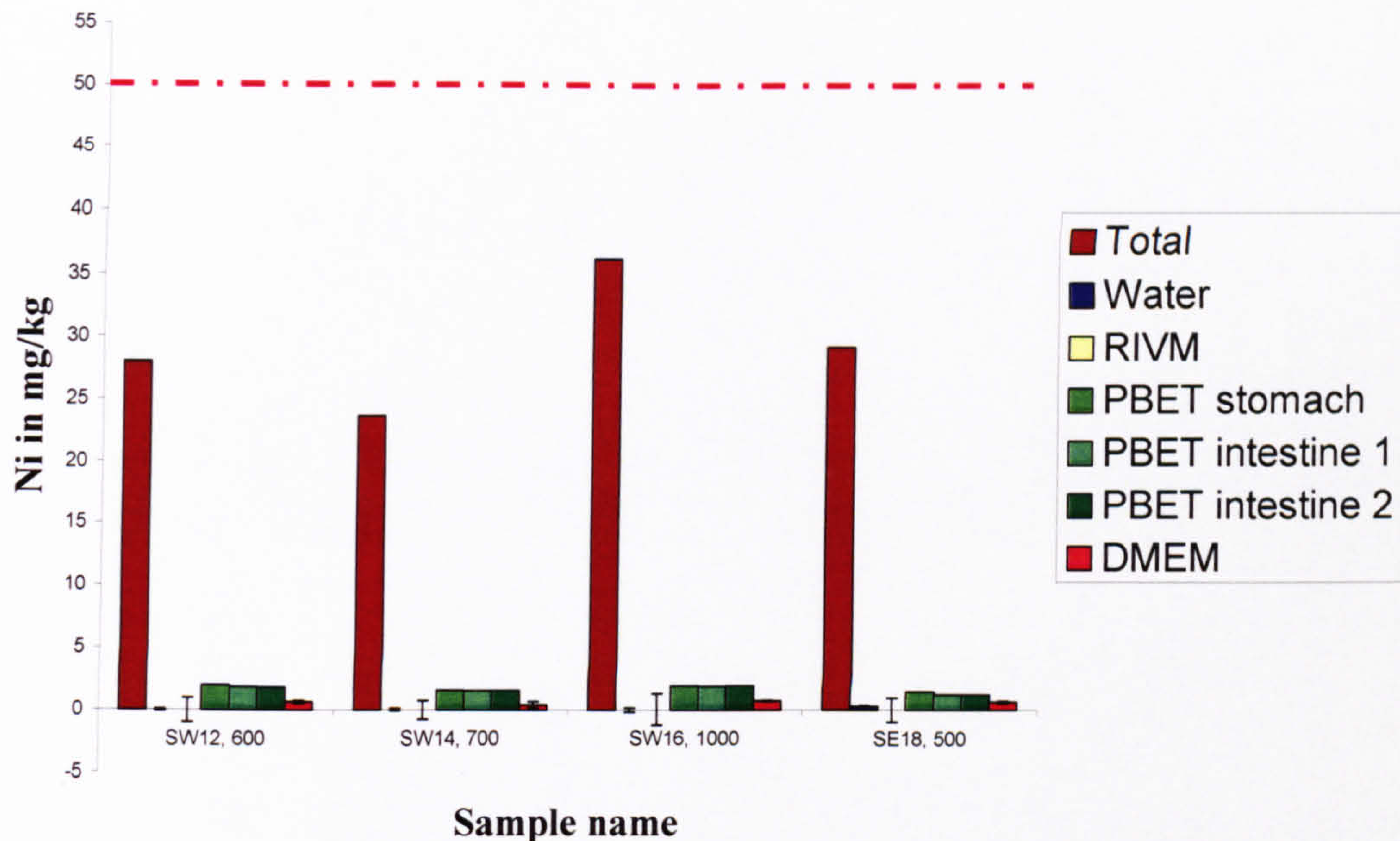


Figure A3.3 - Levels of Ni in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed red line indicates guideline value for Ni – 50 mg/kg.

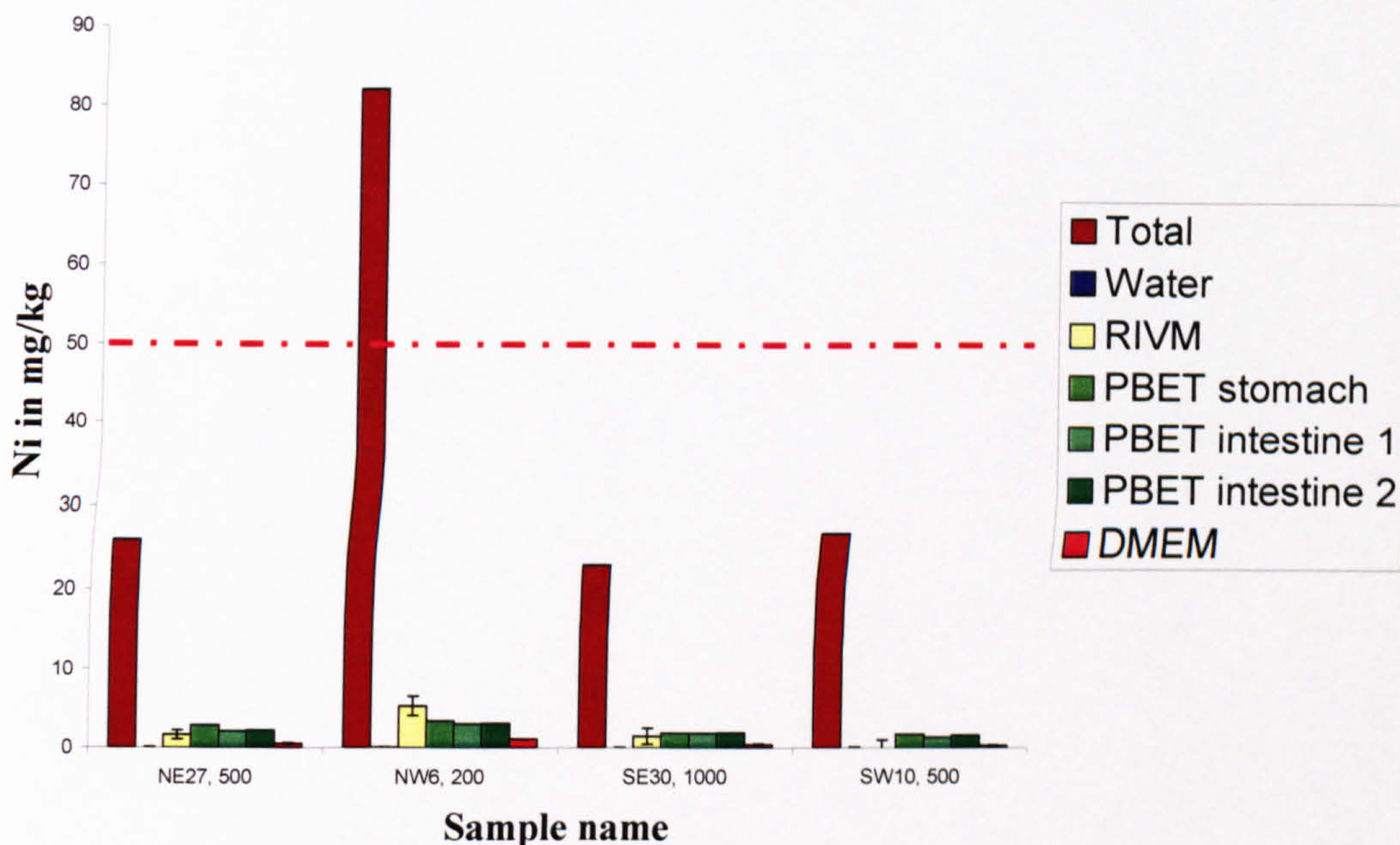


Figure A3.4 - Levels of Ni in 4 contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed red line indicates guideline value for Ni – 50 mg/kg.

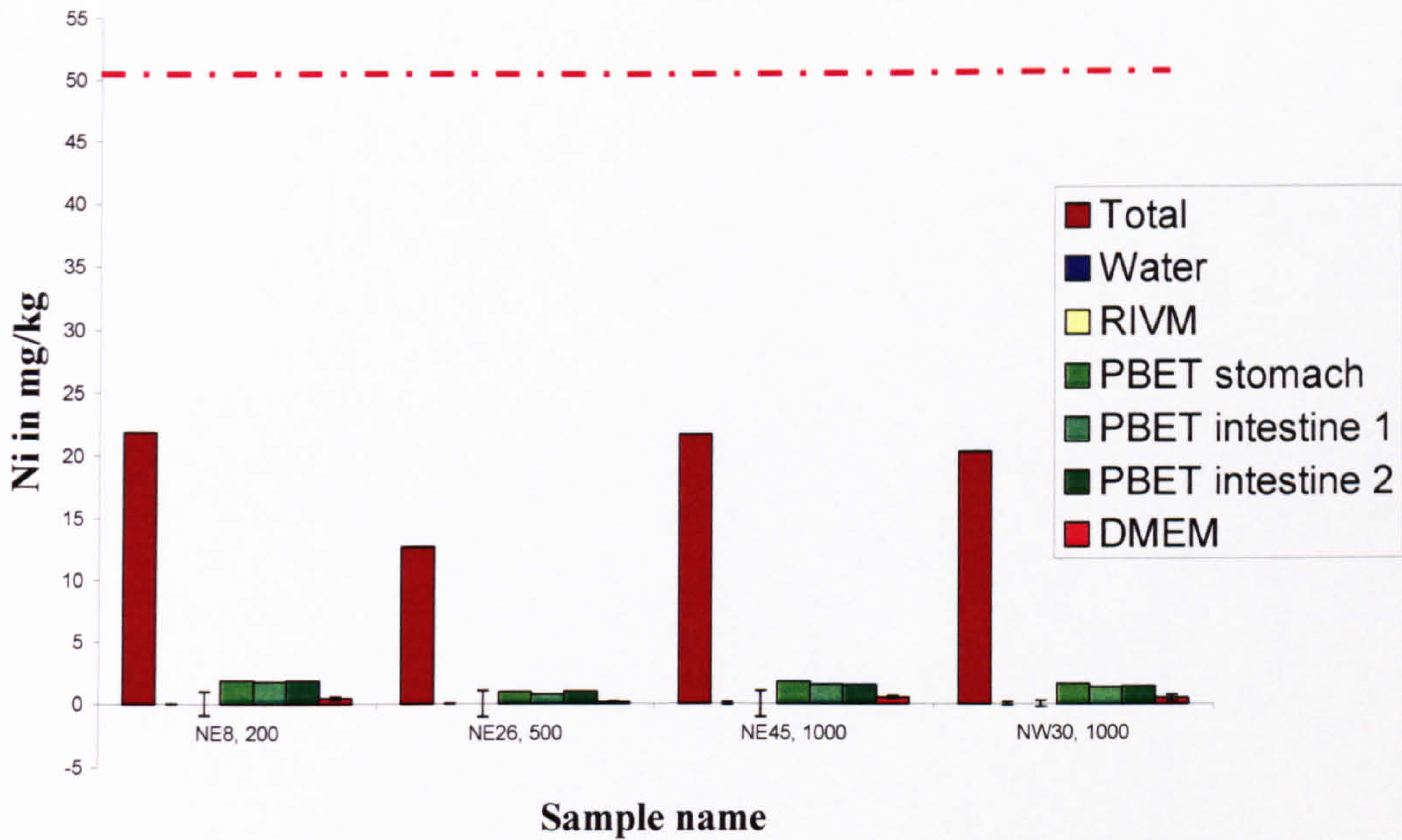


Figure A3.5 - Levels of Ni in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed red line indicates guideline value for Ni – 50 mg/kg.

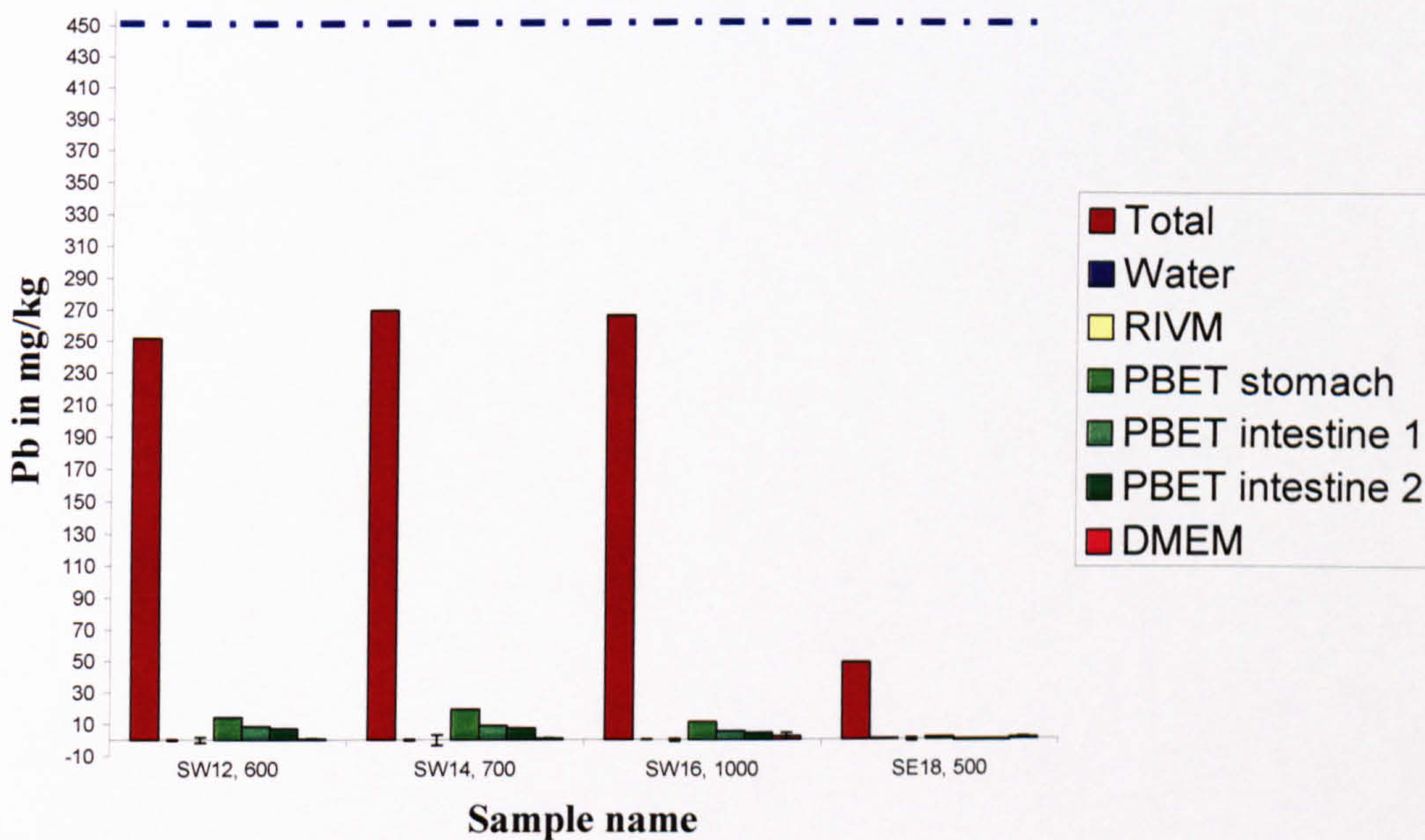


Figure A3.6 - Levels of Pb in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed blue line indicates guideline value for Pb – 450 mg/kg.

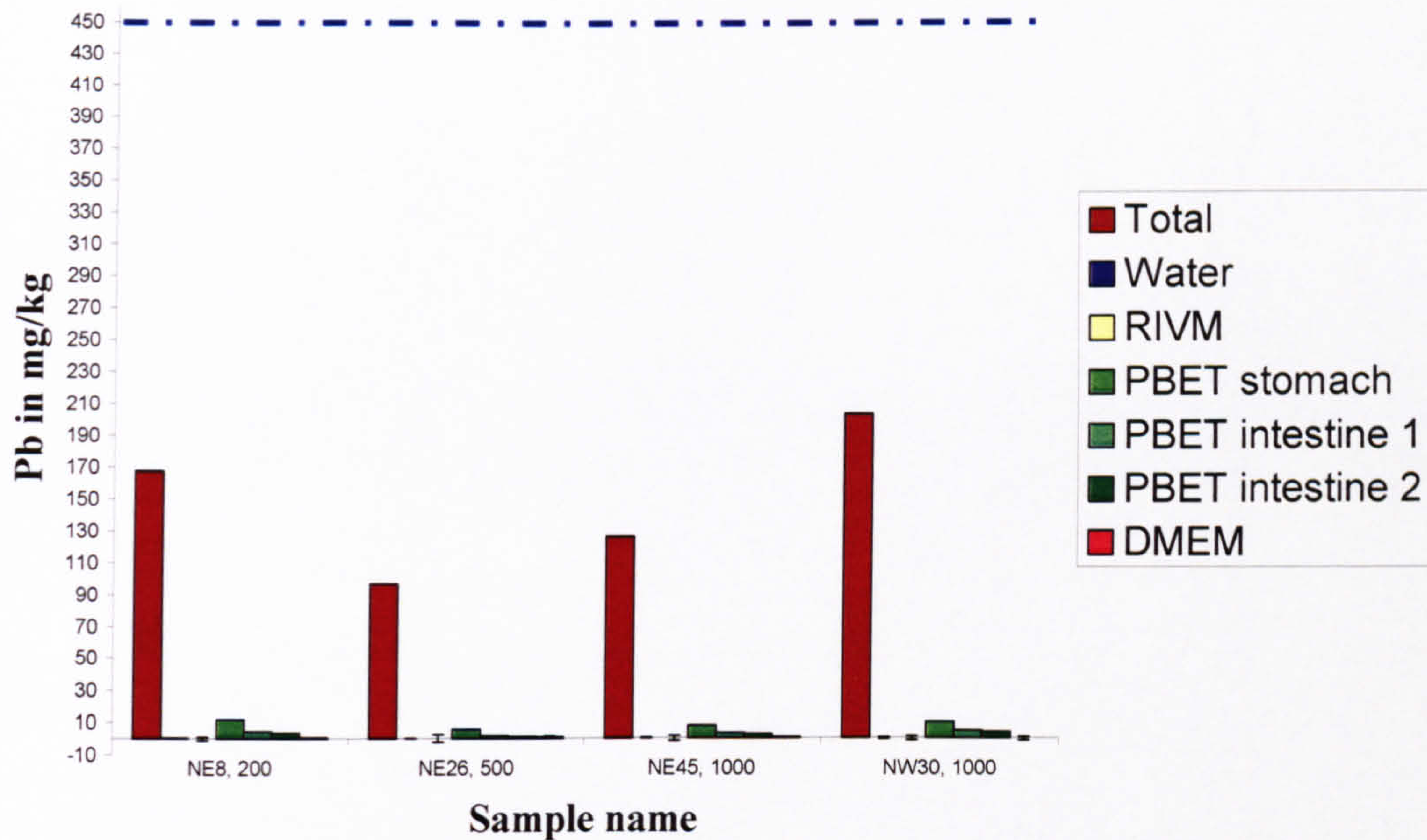


Figure A3.7 - Levels of Pb in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed blue line indicates guideline value for Pb – 450 mg/kg.

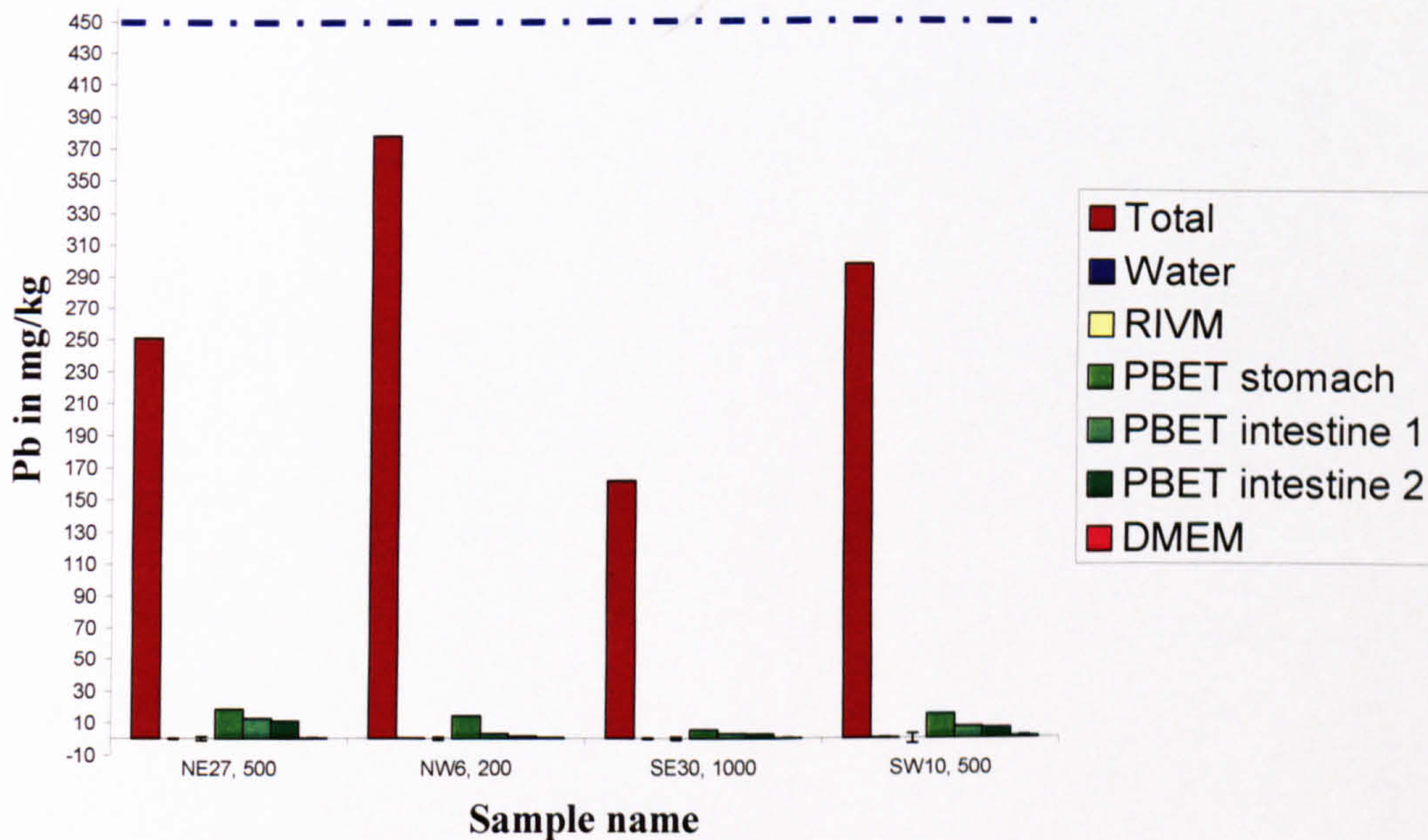


Figure A3.8 Levels of Pb in 4 contaminated samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed blue line indicates guideline value for Pb – 450 mg/kg.

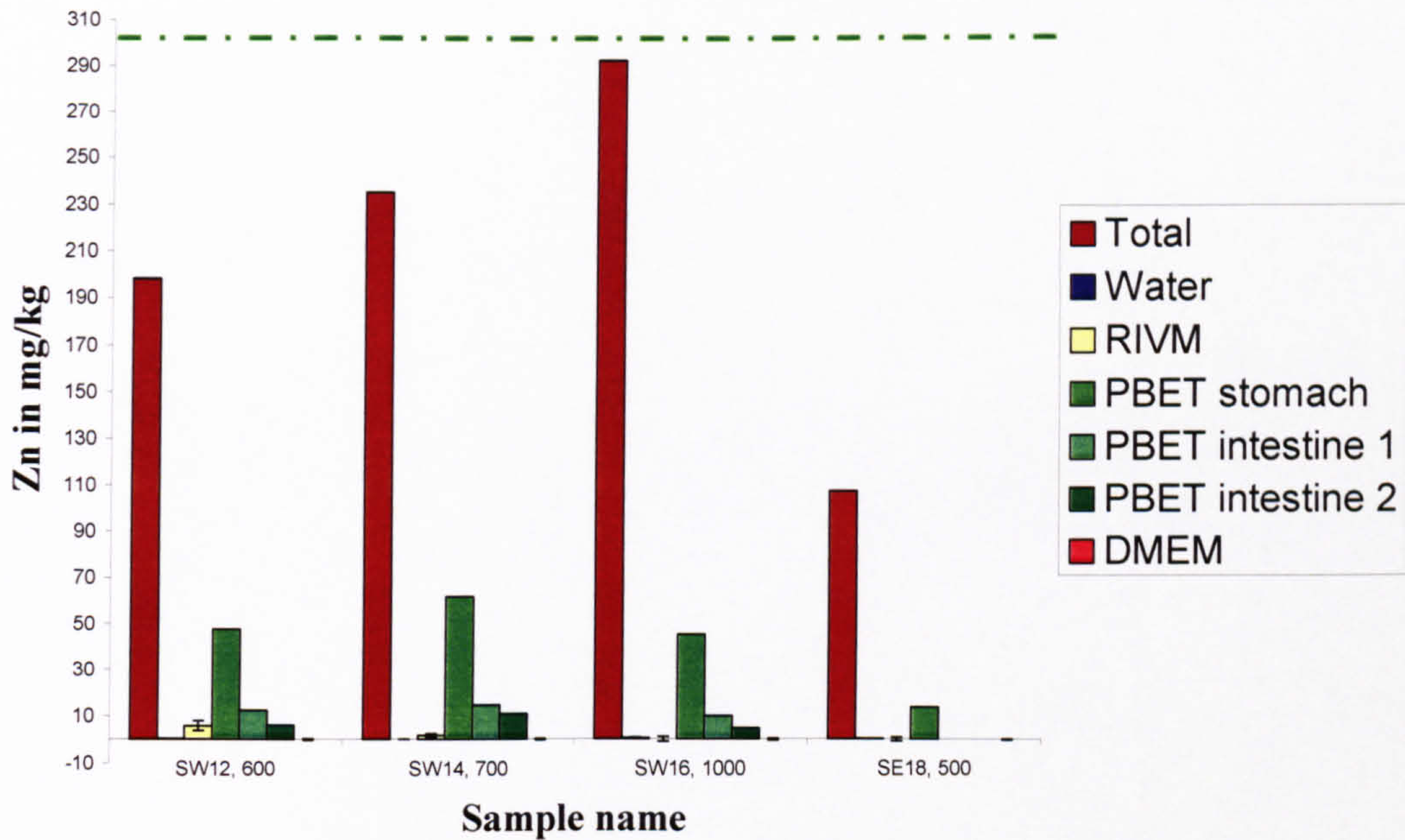


Figure A3.9 - Levels of Zn in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed green line indicates guideline value for Zn – 300 mg/kg.

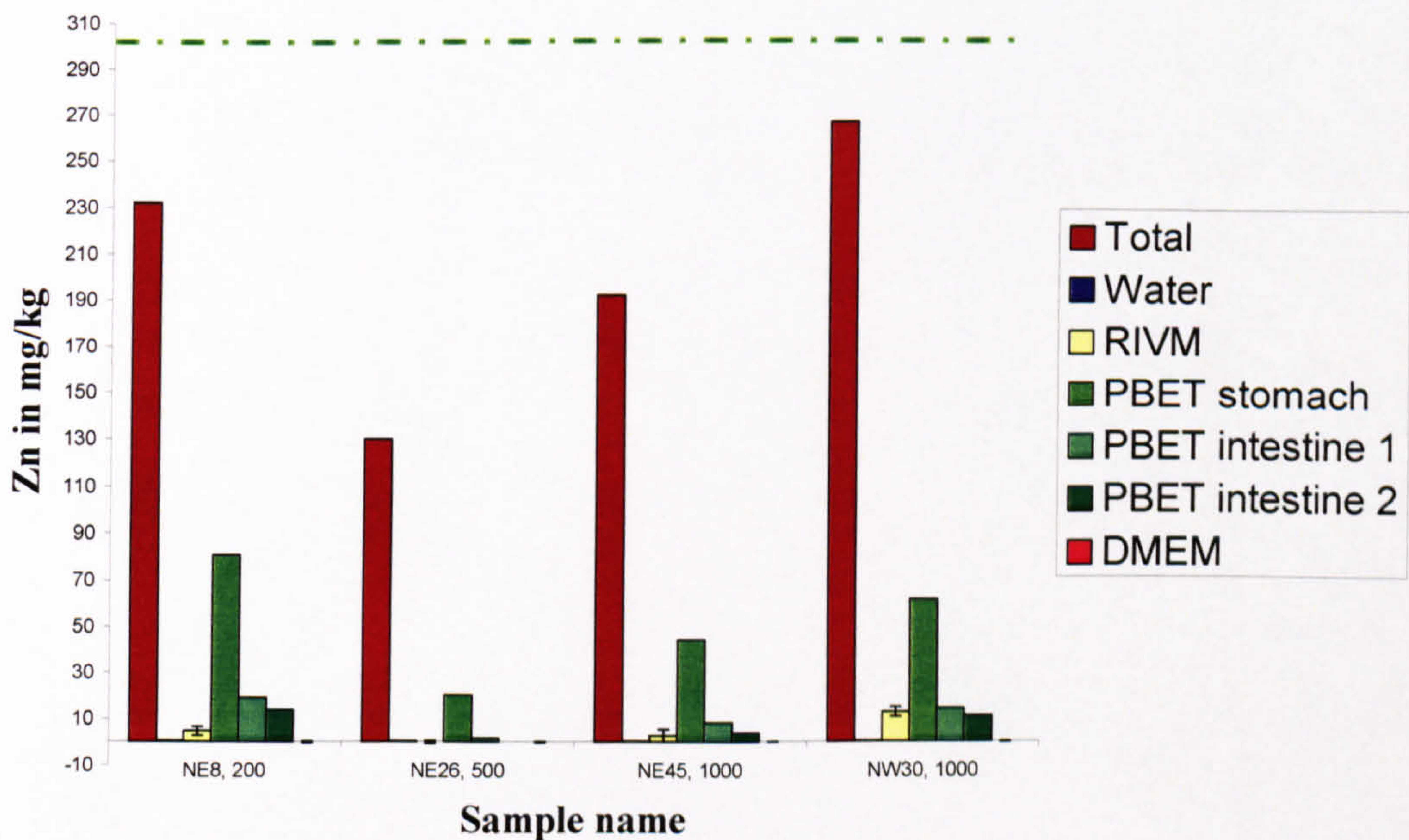


Figure A3.10 Levels of Zn in 4 "control" samples in mg/kg. Total metal (brown); water extract (blue); RIVM extract (yellow); PBET stomach (bright green), intestine1 (khaki), intestine 2 (dark green); DMEM (red). Dashed green line indicates guideline value for Zn – 300 mg/kg.

Appendix 4

Phase contrast microscope picture of HepG2 cells exposed to copper

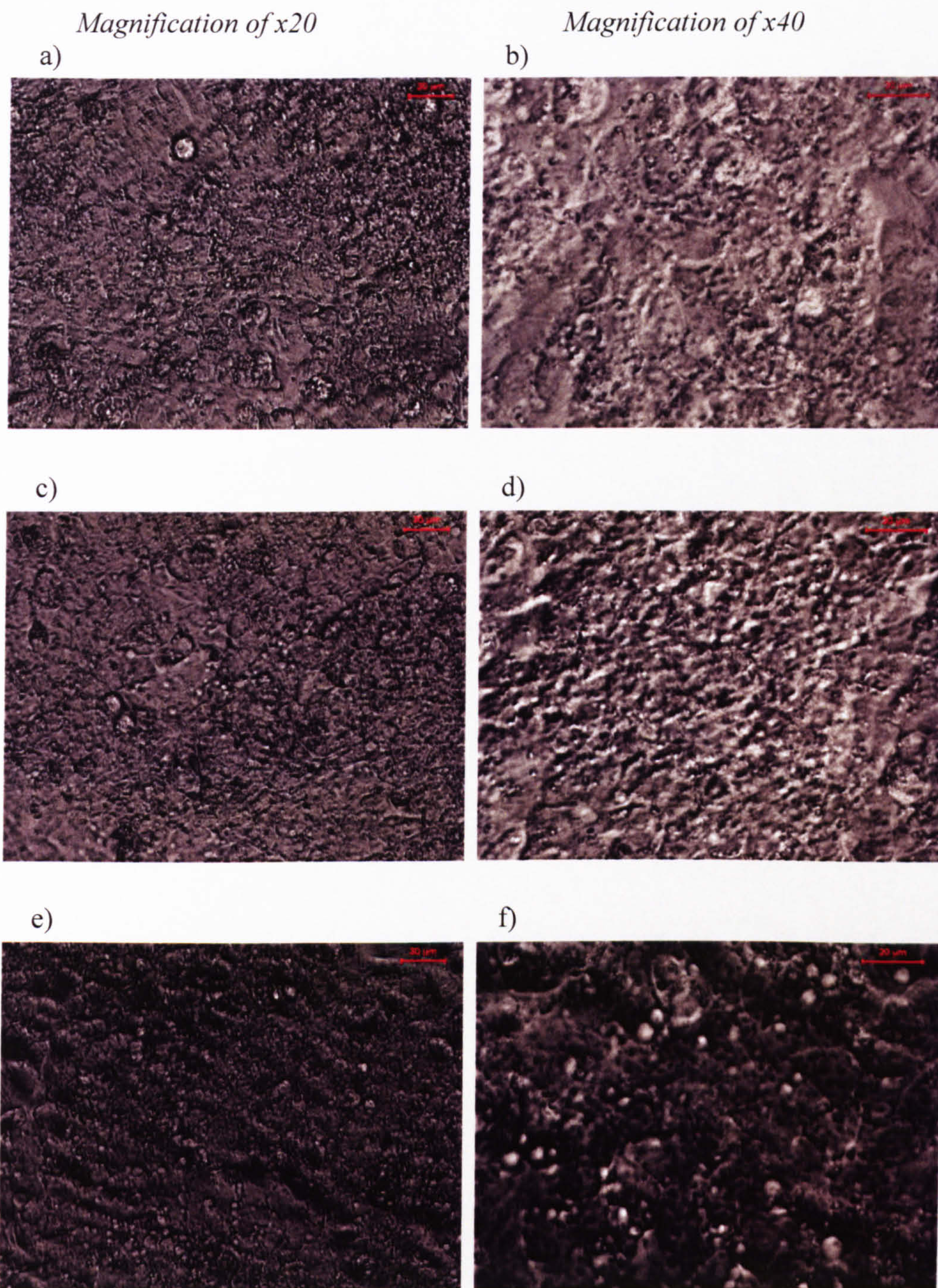
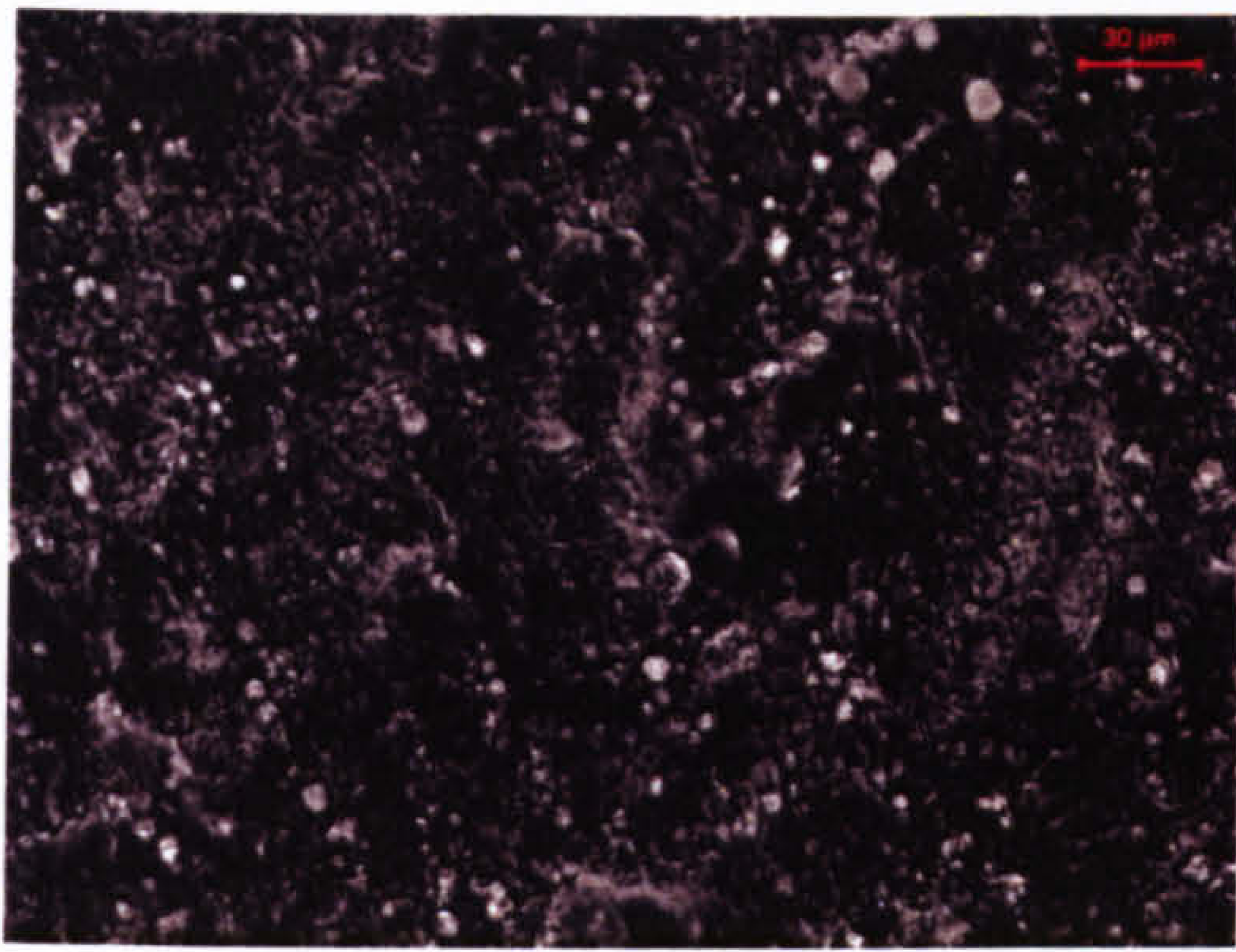


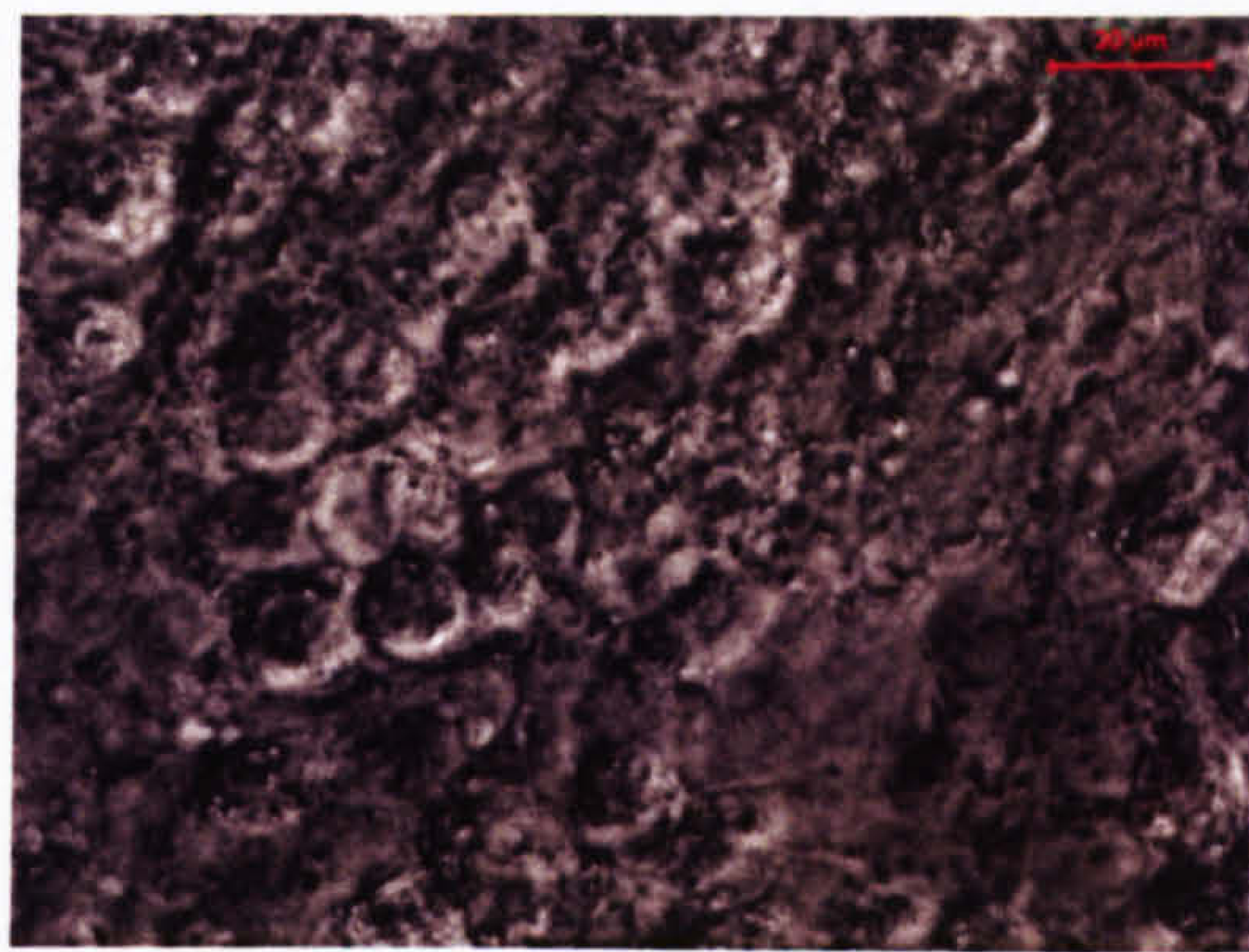
Figure A4.1
a) and b) Untreated "control"HepG2 cells
c) and d) HepG2cells treated with 0.1 mg Cu/L
e) and f) HepG2cells treated with 1 mg Cu/L

Magnification of x20 *Magnification of x40*

a)

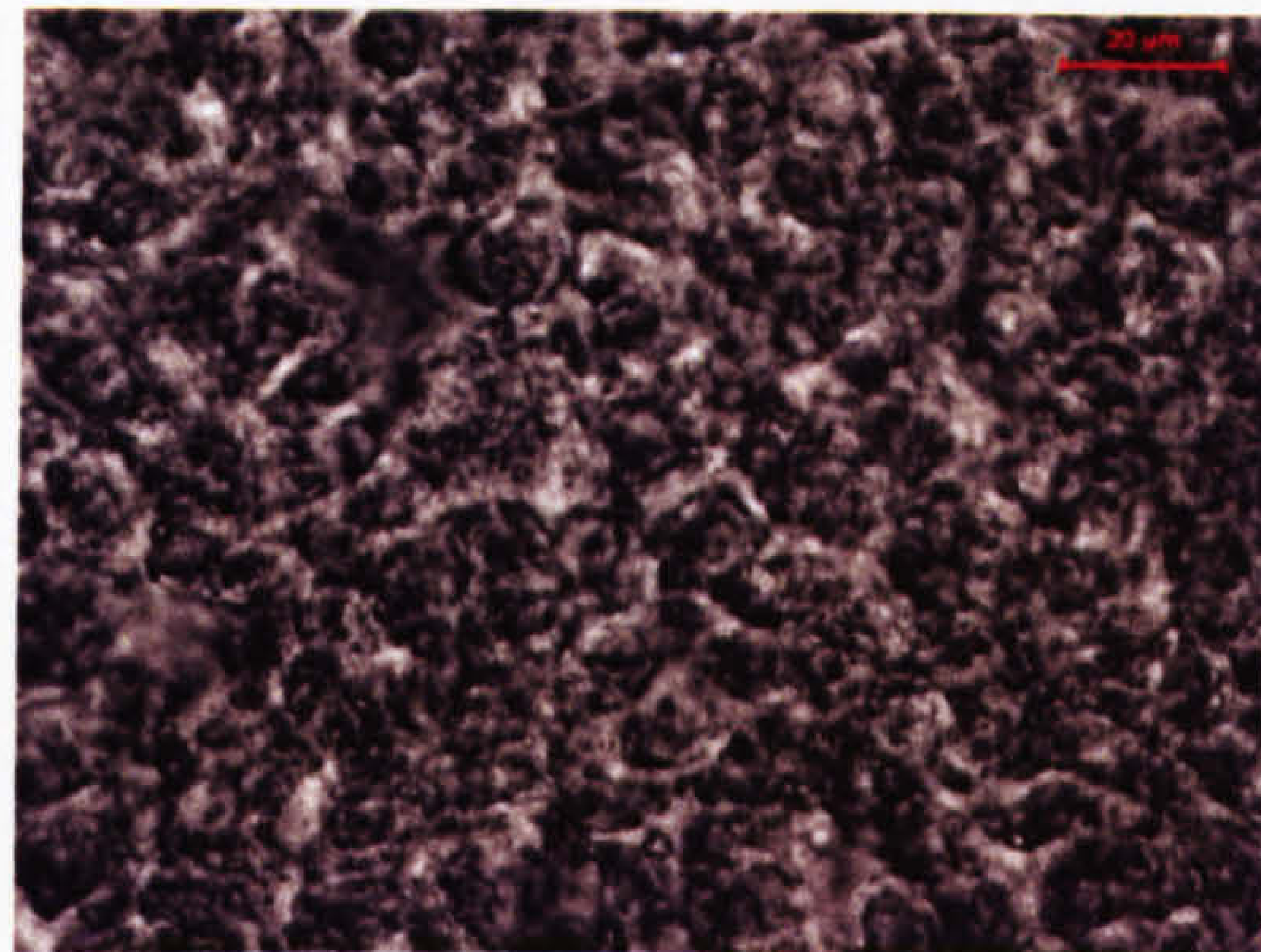


b)

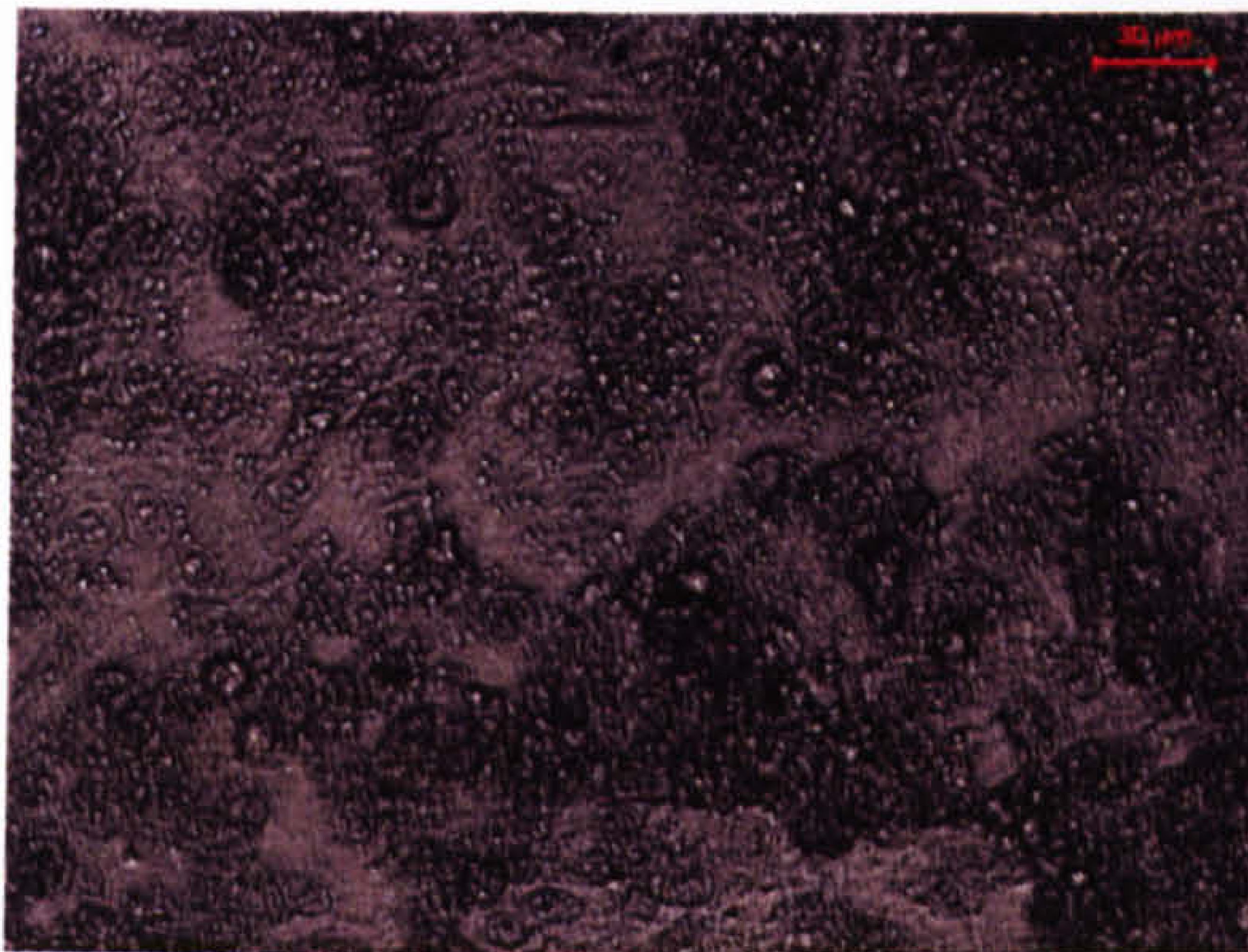


c)

d)



e)



f)

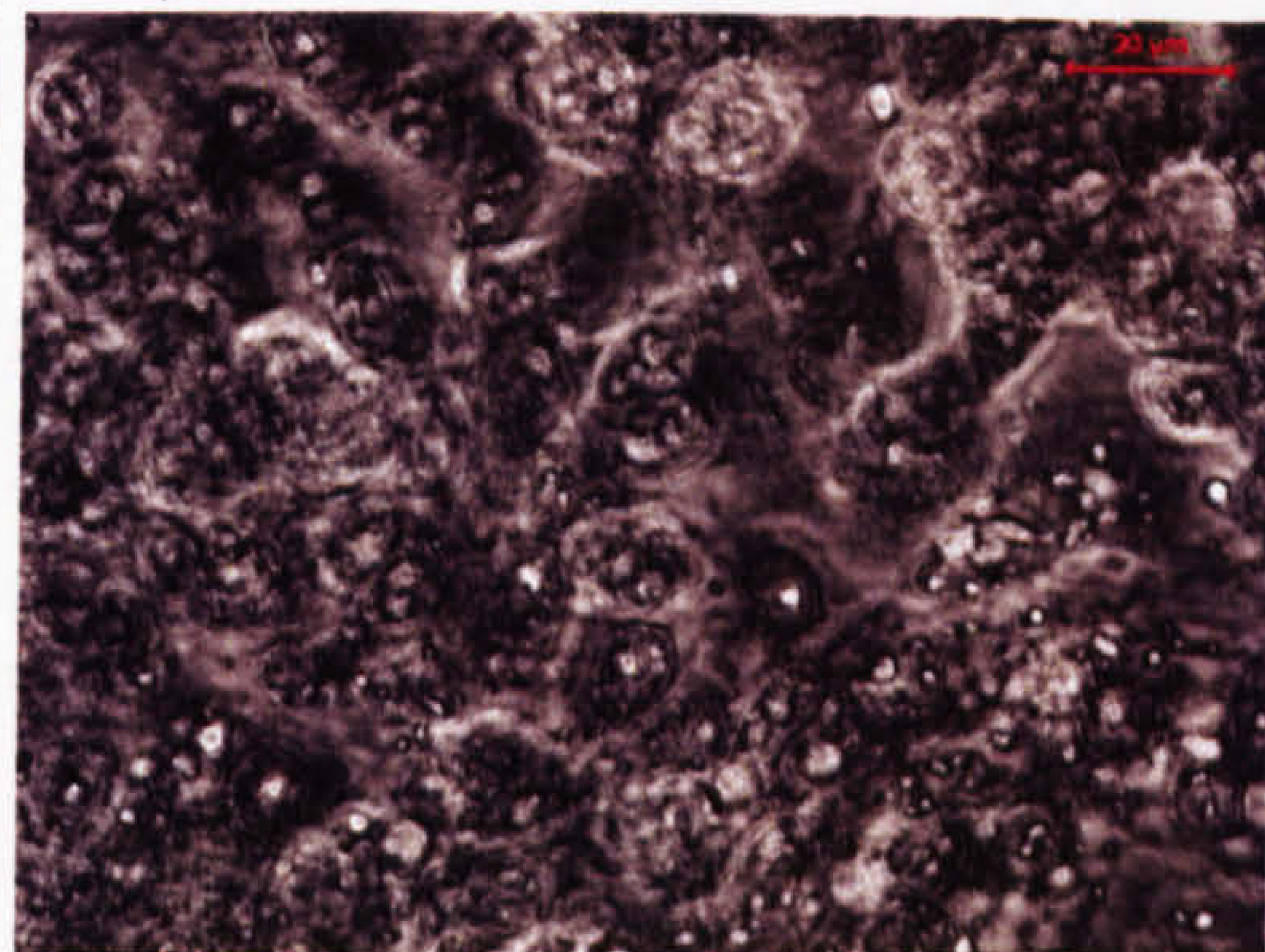


Figure A4.2
a) and b) HepG2 cells treated with 10 mg Cu/L
c) and d) HepG2 cells treated with 50 mg Cu/L
e) and f) HepG2 cells treated with 100 mg Cu/L

Although pictures such as these cannot be used to give quantitative answers to how a treatment affects the cells, they do show interesting qualitative patterns and can provide an indication of the general health of the cell population. The confluency of all cell populations is between 80% and 90%. Figures A4.1 (a) and (b) show "control" healthy, untreated HepG2 cells at two magnifications (x20 and x40); the cells are highly confluent and individual cell shapes are only slightly apparent. As the concentration of copper treatment increases (A4.1 c-f and A4.2 a-f), so the cell shapes become increasingly apparent and more spherical. As the cells become more "stressed" they begin to lift away from the growth surface – hence the round shape and progressively more small white fragments appear in the medium.

Figures A4.3 and A4.4 show HepG2 cells (at two magnifications) subjected to increasing concentrations of zinc. The confluency of all cell populations is between 80% and 90%. A4.3 (a) and (b) show "control" healthy, untreated HepG2 cells; the cells are highly confluent and individual cell shapes are only slightly apparent As the concentration of zinc treatment increases (A4.3 and A4.4), as with cells treated with copper, so the cell shapes become increasingly apparent and more spherical. As the cells become more "stressed" they begin to lift away from the growth surface – hence the round shape and progressively more small white fragments appear in the medium. The change in cell morphology, as concentration increases, does appear differently from cells exposed to copper. The very round shape is not apparent at 10 mg/l with copper treated cells, but is with zinc treated cells.

Phase contrast microscope pictures of zinc exposure to HepG2 cells.

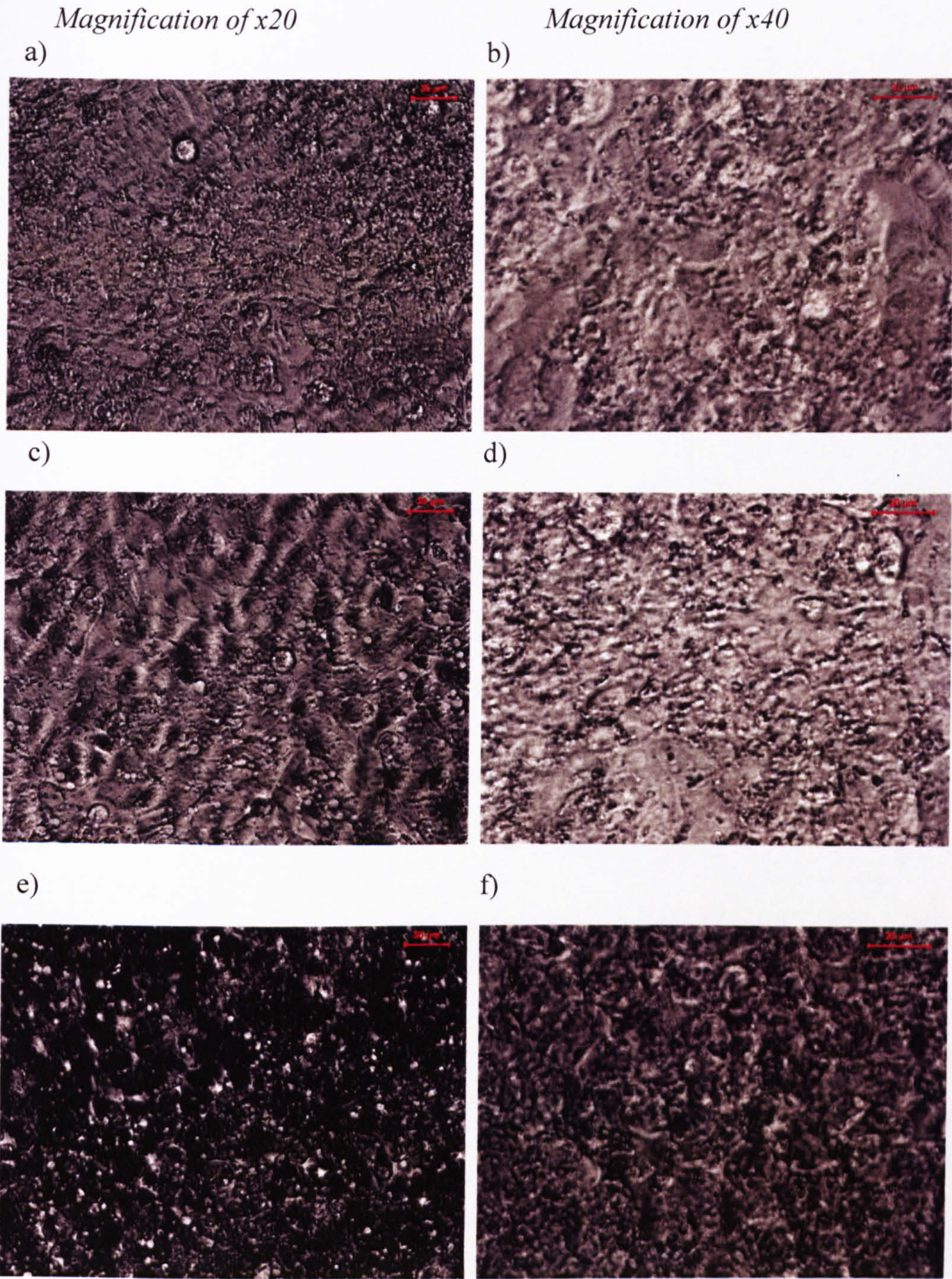


Figure A4.3
a) and b) Untreated "control" HepG2 cells
c) and d) HepG2 cells treated with 0.1 mg Zn/L
e) and f) HepG2 cells treated with 1 mg Zn/L

Magnification of x20 *Magnification of x40*

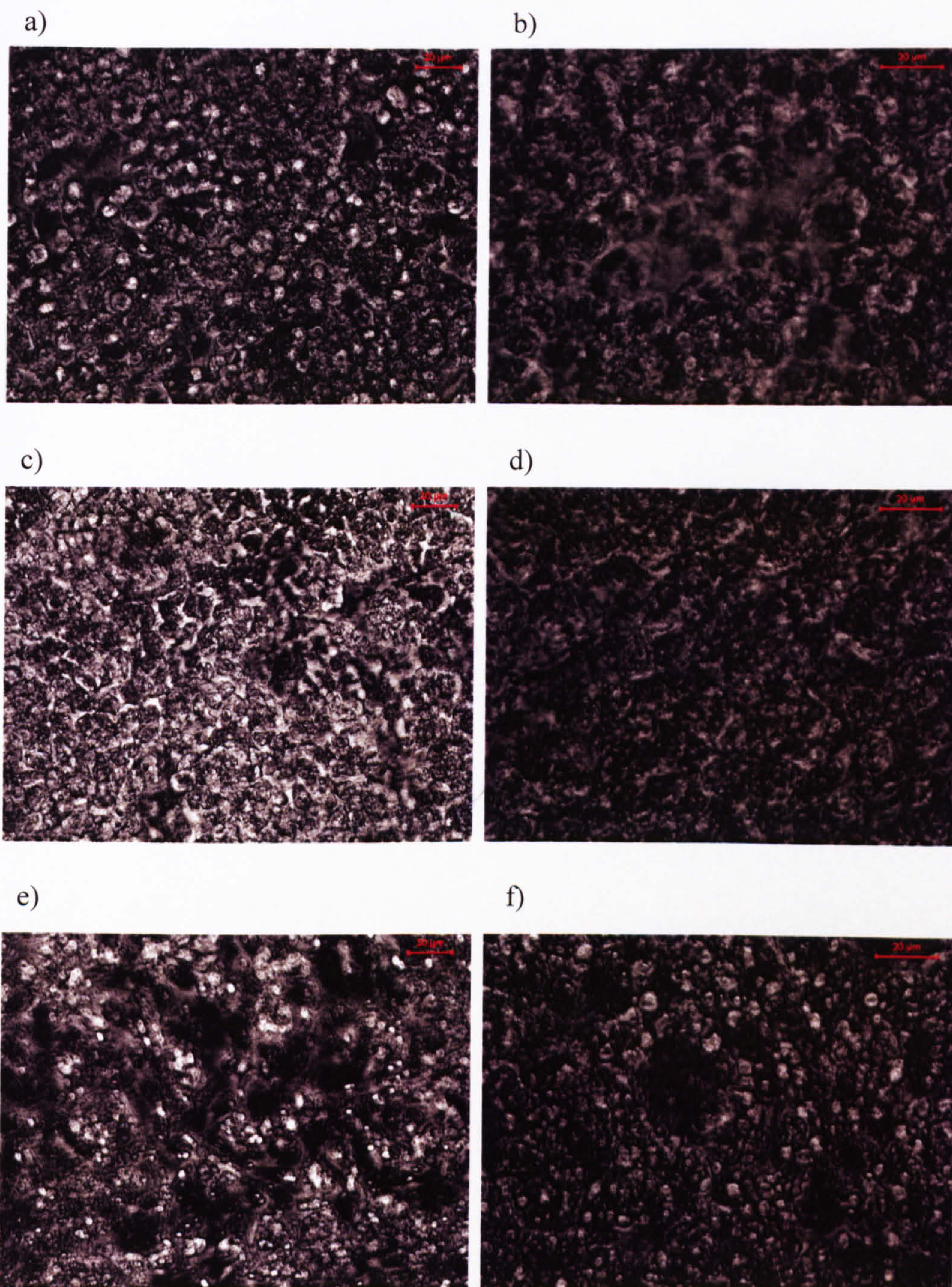


Figure A4.4

a) and b) HepG2 cells treated with 10 mg Zn/L

c) and d) HepG2 cells treated with 50 mg Zn/L

e) and f) HepG2 cells treated with 100 mg Zn/L

Phase contrast microscope picture of HepG2 cells exposed to soil/DMEM extracts

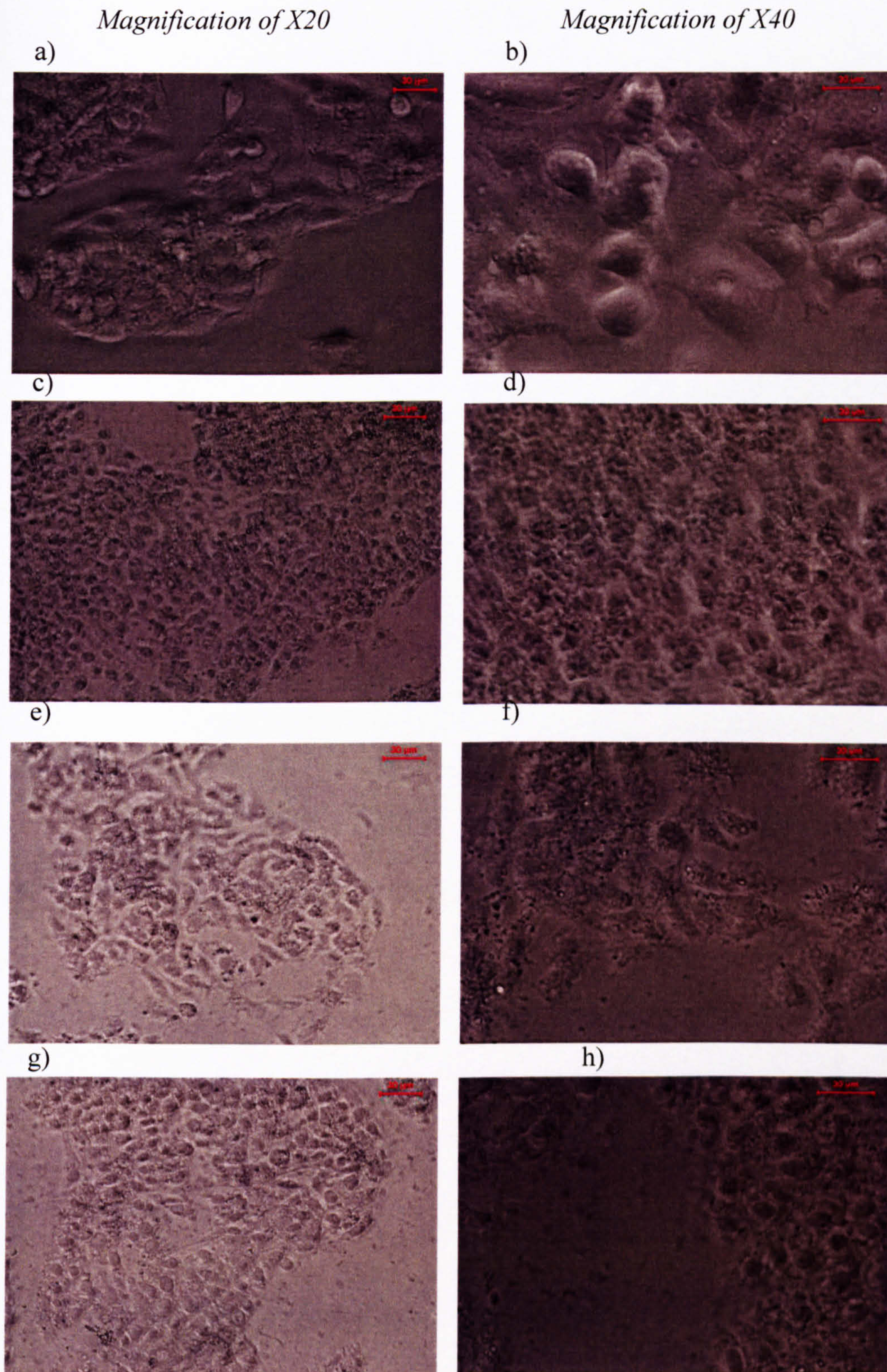


Figure A4.5 a) and b) Untreated HepG2 cells c) and d) HepG2s in irradiated DMEM e) and f) NE26,500/DMEM irradiated g) and h) SW6, 300/DMEM irradiated

The confluency of all cell populations was between 60% and 75%. Figures A4.5 (a) and (b) show "control" healthy, untreated HepG2 cells; the cells are highly confluent and individual cell shapes are only slightly apparent with smooth outlines and the surrounding medium is clear. Figure A4.5 (c) and (d) show HepG2 cells exposed to DMEM, which had had no contact with soil, but had been exposed to γ-irradiation as a sterilisation method. The cells appear rounded, shrunken, and darker and the medium appears to have cell debris in it. Figure A4.5 (e) and (f) show HepG2 cells that have been exposed to soil/DMEM extract that had been sterilised by γ-irradiation. The specific soil (NE26,500), had little/no metal contamination i.e. a "control" soil. Again, the cells appear shrunken and misshapen with cell debris in the medium. Figures A4.5 (g) and (h) also show HepG2 cells exposed to soil/DMEM extract sterilised by γ-irradiation. The soil used was SW6, 300, a soil shown to have high levels of metal contamination and bioavailable copper and zinc. The cells look very similar to those treated with the uncontaminated soil/DMEM extract, cells appear shrunken with substantial cell debris in the medium.

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