THE PHYSICOCHEMISTRY AND TOXICITY OF LANDFILL LEACHATES AND PARTICULATE MATTER

Lata Koshy

A thesis presented for the degree of Doctor of Philosophy

Cardiff University

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School of Biosciences
Cardiff University
Museum Avenue
CARDIFF
CF10 3AX

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For Ma, Pa and Arun

CONTENTS

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DECLARATION

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Koshy, L., Paris, E., Ling, S., Jones, T. and BéruBé, K.A. (2007). Bioreactivity of leachate from municipal solid waste landfills – assessment of toxicity. *Science of the Total Environment* **384**, 171-181.

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ABBREVIATIONS

A1	Landfill A (active & partially contained); Collective sump
A2	Landfill A (active & partially contained); Restored phase
A3	Landfill A (active & partially contained); Active phase
AA	Ascorbic Acid
ABO	α-Benzoin Oxime
ANCOVA	Analysis of Covariance
AOD	Above Ordinance Datum
AP-SA	Alkaline Phosphatase-Conjugated Streptavidin
B1	Landfill B (active,dilute & disperse); Collective sump
B2	Landfill B (active,dilute & disperse); Restored sump
B3	Landfill B (active,dilute & disperse); Leachate reservoir
bp	Base Pairs
BSA	Bovine Serum Albumin
BSE	Back Scatter Electron
C1	Landfill C (restored&contained); Collective sump
C2	Landfill C (restored&contained); Restored phase
C3	Landfill C (restored&contained); Leachate reservoir
C4	Landfill C (restored&contained); Groundwater
cDNA	Complementary DNA
cRNA	Complementary RNA
conc.	Concentration
DCF	2', 7' - Dichlorofluorescin
DCFH	2', 7' - Dichlorodihydrofluorescin
DCFH-DA	2', 7' - Dichlorodihydrofluorescin Diacetate
DEP	Diesel Exhaust Particles
DES	Desferoxamine Mesylate
DMG	Dimethylglyoxime
DNA	Deoxyribonucleic Acid
DTPA	Diethylene Triamine Pentaacetic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
EDX	Energy Dispersive X-ray Microanalysis

Equiv. H_2O_2	Equivalent to H_2O_2 (μM)
ER	Endoplasmic Reticulum
ESD	Equivalent Spherical Diameter
EVOM	Epithelial tissue Volt Ohm Meter
FESEM	Field Emission Scanning Electron Microscopy
GSH	Glutathione (reduced form)
h	Hours
HDPE	High Density Polypropylene
H_2O_2	Hydrogen peroxide
HVCI	High Volume Cascade Impactor
IA	Image Analysis
IC	Ion Chromatography
ICP-MS	Inductively Coupled Plasma – Mass spectroscopy
LFG	Landfill Gas
LOD	Limit Of Detection
MB H_2O	Molecular Biology Grade Reagent Water
MCPP	Mecoprop
MIF	Macrophage Migration Inhibitory Factor
mRNA	Messenger RNA
MSW	Municipal Solid Waste
n	Number of replicates
PM	Particulate Matter
PSA	Plasmid DNA Scission Assay
PUF	Polyurethane Foam
qPCR	Quantitative Polymerase Chain Reaction
QUIPS	Quantimet™ Image Processing Software
RNase	Ribonuclease
RF	Replicative Form
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
ROTAS	Rapid On-site Toxicity Audit System
RTP	Room Temperature and Pressure
RTLF	Respiratory Tract Lining Fluid

SAM	Significance Analysis of Microarrays
SELF	Surrogate Epithelial Lining Fluid
SEM	Scanning Electron Microscopy
SS	Suspended Solids
SSSI	Site of Special Scientific Interest
TBE	Tris-Borate-Ethylenediaminetetracetic Acid
TD_{25}	Dose to cause 25% Total Damage
TD_{50}	Dose to cause 50% Total damage
TDS	Total Dissolved Solids
TEER	Trans-Epithelial Electrical rResistance
tiff	Tag Image File Format
TM	Transition Metals
UA	Uric Acid
UV	Ultraviolet
VOC	Volatile Organic Compounds
v/v	Volume to Volume
w/v	Weight to Volume

ABSTRACT

An understanding of the ranges of toxicity of landfill emissions is crucial in determining the degree of concern we should have about the potential effects they could have upon nearby populations and the surrounding environment. Landfill leachates and airborne PM_{10} were collected and assessed for their transition metal-mediated *in vitro* bioreactivity by a plasmid DNA scission assay (PSA). Human tissue equivalents (EpiDerm™-200 and EpiAirway™-100; MatTek Corp., USA) were exposed to landfill leachate or PM_{10} and evaluated for cell viability (MTT assay) and trans-epithelial electrical resistance (TEER).

The novel use of the oxidant-sensitive probe, DCFH, with landfill leachates was a good indication of leachate ROS activity. The results also revealed that leachate bioreactivity varied inter- and intra-landfills; it was not possible to establish an easily elucidated trend associated with most commonly measured physical parameters (data kindly provided by landfill operators). Metal chelation by EDTA, DTPA, DES and Chelex resin caused significant attenuation of landfill leachate oxidant activity in both the PSA and DCFH assays. Acute leachate toxicity, gauged by a bacterial *V. fischeri* bioluminescence assay (ROTAS™), revealed temporal-dependant hormetic responses from the bacteria which corresponded to high levels of TDS, conductivity and redox potential. Undiluted leachates were not acutely cytotoxic to EpiDerm™-200 following 24h exposure.

PM_{10} was collected from a Cardiff landfill and characterised by FESEM-EDX, ICP-MS and IC. The landfill PM_{10} physicochemistry varied year-on-year, and was dependent upon anthropogenic site activity. Metal chelation by EDTA, DTPA, DES and surrogate epithelial lining fluid caused significant attenuation of landfill PM_{10} oxidant activity in the PSA; this was comparable to Cardiff urban PM_{10} reactivity. The $PM_{2.5\text{-}0.1}$ soluble fraction was the most oxidant component. Landfill PM_{10} (500µg/ml) was not cytotoxic to EpiAirway™-100 following 24h exposure. Preliminary toxicogenomics suggested several chaperones and heat shock proteins were up-regulated by landfill PM_{10}. However, human disease causality was not confirmed.

CHAPTER 1

INTRODUCTION

1.1 RESEARCH OVERVIEW

There are strong national and international concerns about the possible adverse health effects of living in the vicinity of municipal solid waste (MSW) landfills. Quantifying the toxicity of landfill emissions is vital in determining the degree of concern we should have about the potential effects these could have upon nearby populations and the surrounding environment. Landfill leachate and particulate matter from several sources were characterised using a variety of *in vitro* assays: plasmid DNA scission, 2',7'-dichlorodihydrofluorescin fluorescence, *Vibrio fischeri* bioluminescence and 3-D human epidermal and respiratory epithelial models were used to assess the *in vitro* bioreactivity of landfill leachate and particulate matter.

1.2 LANDFILL HISTORY

As the human pre-industrialised population grew and became less nomadic, urbanisation placed increasing demands on the disposal of the rising levels of municipal waste; early civilisations realised the importance of clean sanitisation. Landfilling is the oldest method of waste disposal with the first planned landfill recorded in Crete in 3000 BC, when waste was placed in large pits and covered with soil. By 2000 BC, Greek authorities in Athens opened a municipal waste landfill a mile away from the city walls, and there is evidence of composting in China in the same era (Green-Wilkinson, 2000).

Diseases such as cholera, yellow fever and smallpox were often attributed to the large numbers of vermin that thrived on open rubbish dumps. The plague of 1348-9, also known as the Black Death, killed two-thirds of London's population and was certainly aggravated by poor hygiene levels. Following this epidemic, waste was dumped outside the home; 'raker men' were employed to cart these mounds left on the streets to the city gates and scavenging was rife. The situation did not improve until the late 19th century when local authorities were charged with the duty to arrange for the removal and disposal of household waste (Hibbert, 1988).

1.3 CURRENT STATUS OF UK LANDFILLS

An unavoidable issue, landfills are of utmost environmental importance in the UK, which currently disposes approximately 60% of its waste in landfill sites, decreasing from over 80% in 2005, now placing the UK within the top 15 EU member landfillers (Eurostat, 2009). It provides a relatively cheap method of disposal and efficient utilisation of landfill gas can be a source of energy. Often considered to be the last resort, as it lends itself to an "Out of Sight, Out of Mind" philosophy, modern day landfilling is a highly regulated activity, with current legislation promoting alternative methods of disposal – recycling, composting and 'Energy From Waste' incineration (Defra, 2004).

1.3.1 WHAT IS WASTE?

"Waste shall mean any substance or object....which the holder discards or intends to, or is required to discard" (European Council Directive 75/442/EEC). However, in many cases, what constitutes waste is not clear, as recycling technologies improve and it becomes increasingly difficult to assign end-of-life criteria to materials. The breakdown of all waste streams currently produced in the UK is depicted in Figure 1.1.a. Agricultural waste consists mostly of farmyard slurry, manure and crop residues, and is usually spread onto land. The mining and quarrying, and construction and demolition sectors can often produce hazardous wastes that cannot be disposed alongside municipal solid waste (MSW). Although sewage sludge from waste water treatment residue is high in nutrient content, the amount going into landfills is controlled, corresponding to 1.5 million tonnes per annum (Figure 1.1.a; Defra, 2006). Certain wastes from commercial and industrial sectors can also be placed in non-hazardous landfills, however significant amounts of minerals and chemicals cannot be sent to MSW landfills.

In the UK, MSW is defined as waste collected by, or on behalf of, Local Authorities (Defra, 2004). This can include paper, cardboard, food, glass, plastics, metals, wood and textiles. During 2007-08, Wales produced 1.8 million tonnes of municipal waste. Sixty five percent (1.165 million tonnes) of this

came from households, with the remainder coming from the commercial sector, street sweepings, park wastes and litter. There was a decrease by 2% of MSW in Wales from 2006-07 to 2007-08, and this was accompanied by a 8% decrease in the amount going to landfills (National Assembly for Wales, 2008). Yet, over 60% of Welsh MSW still gets placed in landfills (Figure 1.1.b), making waste disposal a leading environmental issue.

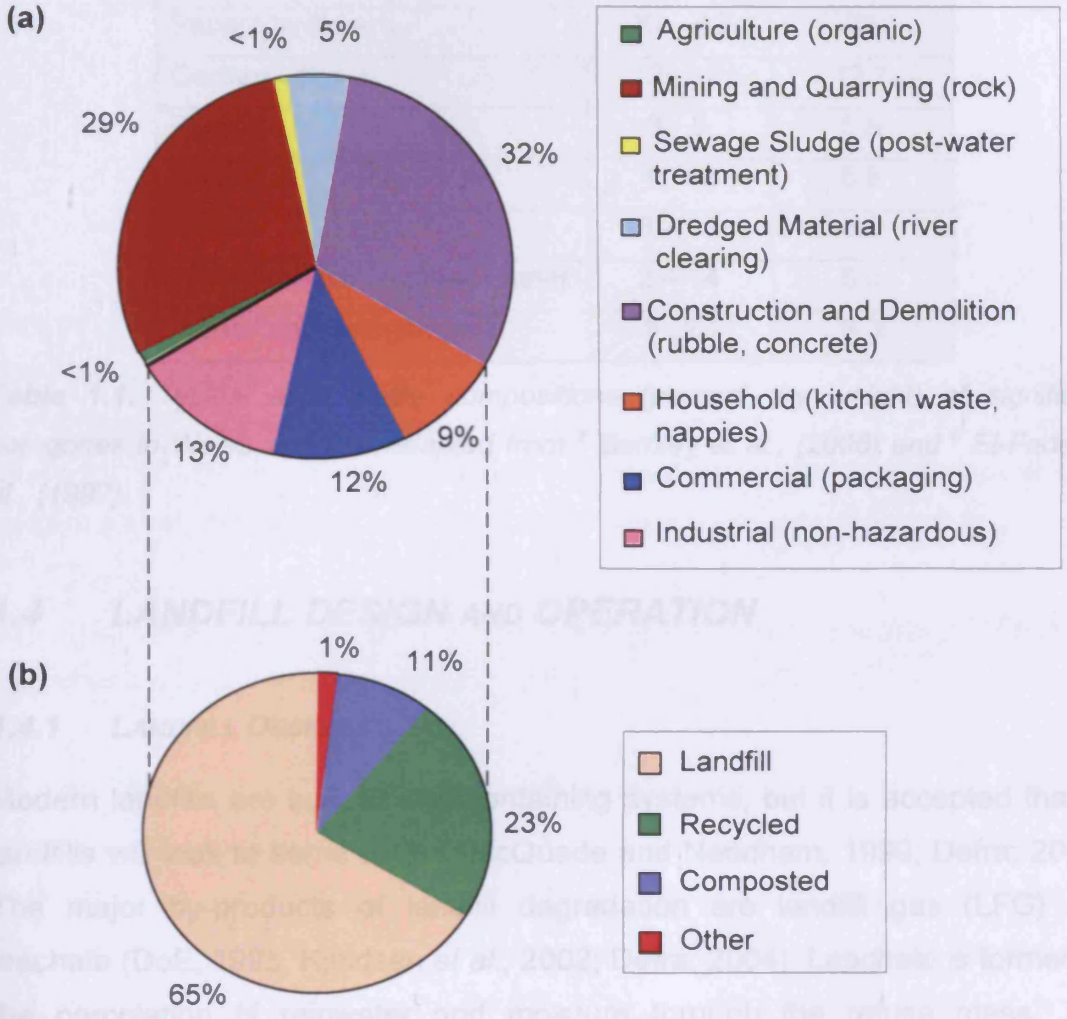

Figure 1.1: *Percentage by mass of* ***(a)*** *The 8 major categories of waste production and the routes of disposal of non-hazardous industrial, commercial, household and industrial waste in the UK, 2004 (Defra, 2006);* ***(b)*** *Municipal waste management in Wales 2007 – 08 (National Assembly for Wales, 2008).*

The wide range of waste categories that typically constitute MSW in the EU is categorised in Table 1.1. Wales produces very similar amounts of the major

waste streams, although the amount of Welsh food waste is below the lower end of the scale in comparison to the rest of the EU. The composition of MSW is highly variable, depending on a range of factors including population density, seasonality, economic status and the waste minimisation strategies in operation.

WASTE CATEGORY	EU [b]	WALES [a]
Food Waste	20 – 50	15.7
Paper/Cardboard	20 – 42	22
Garden Litter	12 - 18	12.7
Plastics	3 - 8	5.8
Glass	4 - 12	5.8
Metals	3 – 13	6.6
Wood/Rubber/Textiles/Leather	2 – 14	6.5
Inerts/Other Inorganics	1 - 20	8.3

Table 1.1: *Typical solid waste compositions (percent dry weight) of significant categories in Wales and EU. Adapted from [a] Burnley et al., (2006) and [b] El-Fadel et al., (1997).*

1.4 LANDFILL DESIGN AND OPERATION

1.4.1 LANDFILL DESIGN

Modern landfills are built as self-containing systems, but it is accepted that all landfills will leak to some extent (McQuade and Needham, 1999; Defra, 2004). The major by-products of landfill degradation are landfill gas (LFG) and leachate (DoE, 1995, Kjeldsen *et al.*, 2002; Defra, 2004). Leachate is formed by the percolation of rainwater and moisture through the refuse mass. This migrates down through void space within the waste mass, drains away in the engineered drainage layer and collects at the lowest point in a sump or lagoon (Figure 1.2). LFG - largely comprising CH_4 and CO_2 - is produced by the bacterial metabolism and degradation of organic wastes in the landfill. This must be pumped or vented-off (Figure 1.2), to avoid uncontrolled release of trace toxins within the gas (Williams, 2002). Although the CH_4 in LFG can be

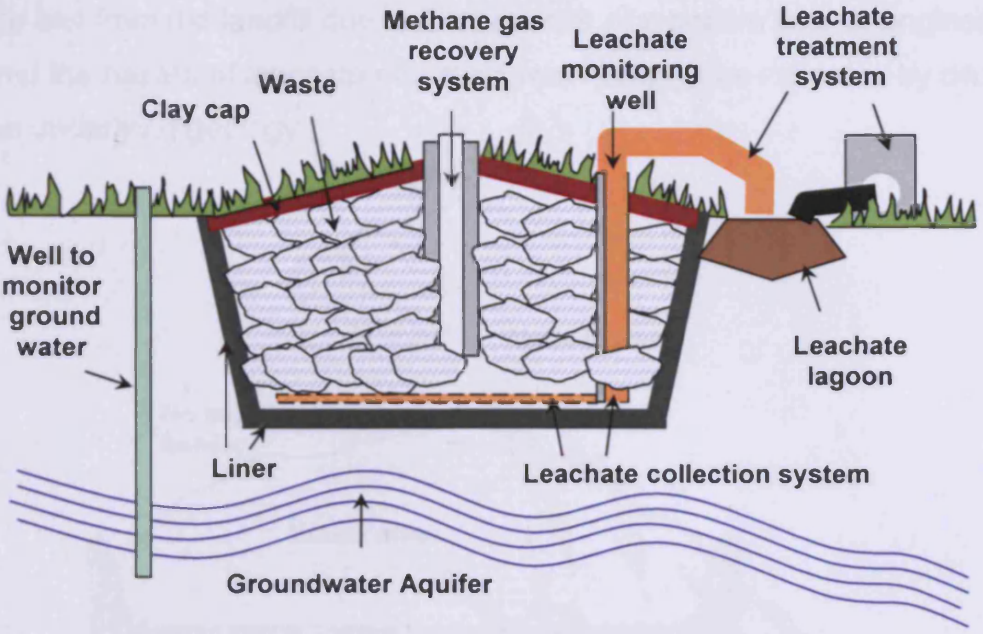

Figure 1.2: *A cross-section of a modern containment landfill, depicting the key components. Waste is placed in the lined landfill and covered. Leachate is collected at the bottom of the landfill before being pumped up into lagoons. Gas is collected for flaring or energy use. Local aquifer groundwater is monitored for contamination. Leachate treatment systems aim to reduce the pollutant levels in leachate.*

extracted for energy recovery, it is also highly flammable, thereby conferring a potential explosion and fire hazard (El-Fadel *et al.*, 1997).

Current legislation (The Landfill [England and Wales] Regulations, 2002) of new landfills requires using the energy from this waste product by flaring-off and powering generators (Defra, 2004). A network of boreholes surrounding the landfill site is used for groundwater monitoring of underlying aquifers (Figure 1.2). The evolution of landfill design has produced the two major designs seen today – the 'dilute and disperse' and 'containment' landfills (Figures 1.3 and 1.4).

1.4.1.1 Dilute and Disperse

Based on the theory of natural attenuation, the 'dilute and disperse' landfill was the original type of unlined landfill used before most current design guidelines and legislation were introduced. This was commonly seen in sites from the

1970s and 1980s (Figure 1.3). There was no controlled regulation of water entry or exit from the landfill due to the absence of a bottom liner or engineered cap; and the hazard of leachate migration was hoped to be mitigated by dilution into the underlying geology.

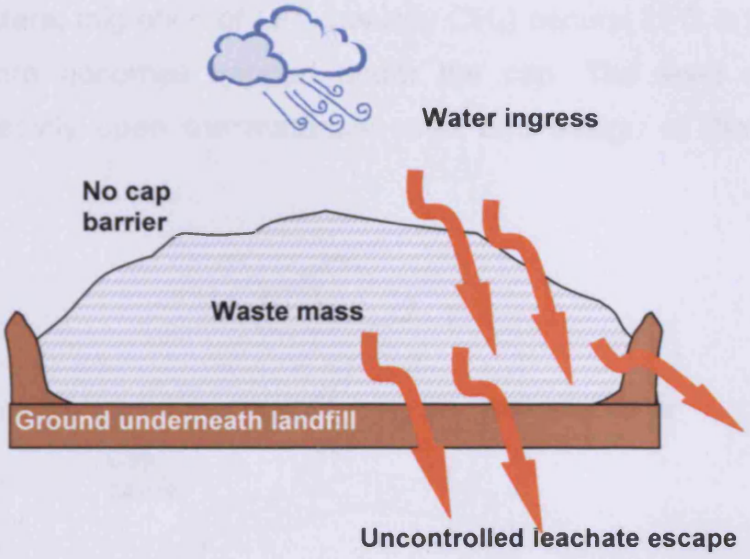

Figure 1.3: *A dilute and disperse landfill design illustrating leachate leakage. There is minimal control of water entry and exit from the landfill, resulting in an increased risk of leachate escape.*

Natural attenuation is the result of naturally occurring physical, chemical and biological processes remediating the pollution in the groundwater released from the site. Microorganisms in the leachate plume metabolise the contaminants by transferring electrons to minerals and compounds that are present in the natural geology (e.g. ferric oxides and sulphates in the groundwater). The former, results in soluble ferrous oxides, and the latter, often forms sulphides (e.g. H_2S, FeS). Ferrous oxides can be used as a tracer for leachate plumes (Christensen *et al.*, 2001). Dilute and disperse is the most basic form of landfill and still seen in developing countries, but in the UK, the Pollution, Prevention and Control Directive requires that groundwater is protected from landfills (Defra, 2000).

1.4.1.2 CONTAINMENT

The 'containment design' is the most expensive and common type of landfill

system operating in the UK today. A liner system is used to contain the movement of leachate before it is pumped out of the site (Figure 1.4). A cap is built over the top of the finished site to prevent infiltration of rainwater, and thus minimise leachate formation. This also traps LFG, which must have a separate system of pipes for collection at the surface. The more impermeable the cap, the more lateral migration of LFG (usually CH_4) occurs; LFG is lighter than air, and therefore becomes trapped under the cap. The level of containment depends heavily upon the materials used and design of the landfill (Allen, 2001).

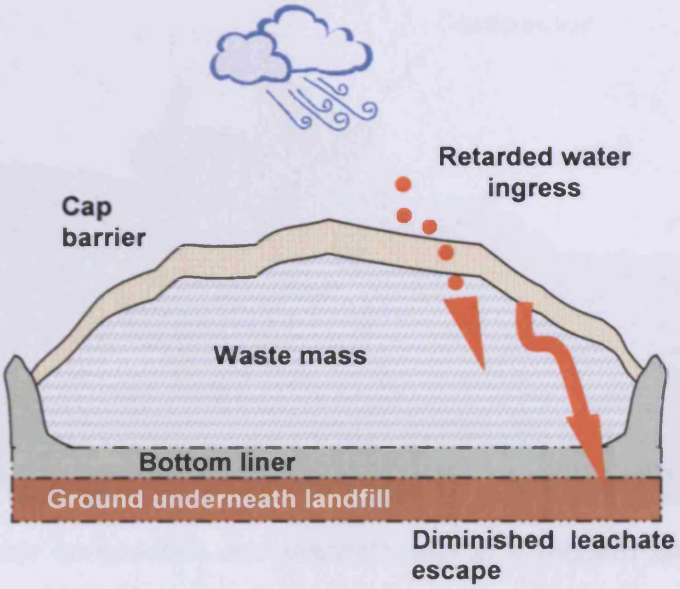

Figure 1.4: *Containment landfill with an engineered cap and liner. Water ingress is reduced with the aim of minimising leachate production.*

1.4.2 LANDFILL OPERATIONS

Within a modern landfill, solid waste is deposited sequentially into cells and compacted to 0.8-1 tonne/m^3 by specialised spiked steel wheel compactors (Figure 1.5; Sormunen *et al.*, 2008). The active cell is covered with soil at the end of each workday. The cellular nature of modern landfills allows greater control over the predicted settling within the waste mass, and soil cover on the waste mass helps minimise the attraction to vermin birds, flies, rats and other pests (The Landfill [England and Wales] Regulations, 2002; Brabham *et al.*, 2006).

In addition to landfills, there are also landraises, in which the waste is laid on the ground rather than within it. Leaks are easier to repair and the waste may be kept further away from groundwater sources, although this is site specific (Strange, 2002). Although landraises are quite common in the USA, they are less so in the UK. The major concern tends to be the visual intrusion into the landscape; nonetheless, landraises are easier to construct and maintain than landfill sites.

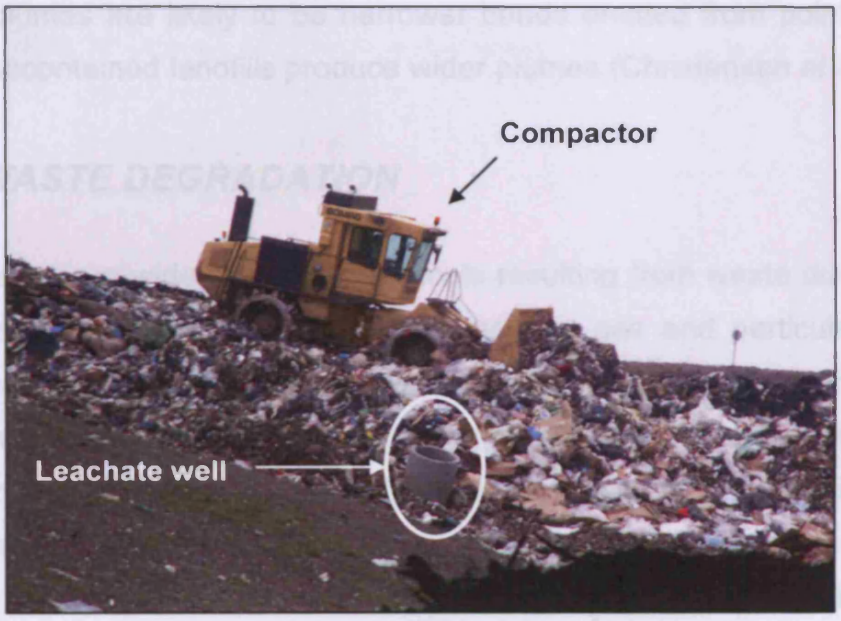

Figure 1.5: *Waste compaction and leachate well in a modern landfill. Image by L. Koshy.*

1.4.2.1 Leachate Collection Systems and Management

All landfill liners are likely to eventually leak (Environment Agency, 1995; Westlake, 1997; Allen, 2001). The cellular, compacted nature of MSW landfills means that the zones of saturation may not follow a directly vertical route down through the waste and different parts of the landfill will develop leachate at different rates (Sormunen *et al.*, 2008).

Although often wrapped by a synthetic geotextile, the perforated leachate collection pipes can still clog with sediment within 10 years, whereas the anticipated active waste degradation phase of a landfill could be 50 years or more (Westlake, 1997). Pipes can crack or collapse due to a number of

reasons including chemical attack by leachate, microorganisms producing destructive metabolites, precipitation of minerals, silt accumulation and differential settlement as the waste degrades. All these processes lead to a build-up of the leachate head and an increase in pressure, which eventually causes leaks through the liner systems (Allen, 2001). Older waste sites that operate to out-dated practices may generate toxic leachates, leading to plumes of contamination, especially as less stringent engineering standards were in existence during construction. Due to the engineering of modern landfills, leachate plumes are likely to be narrower bands emitted from point sources, whereas uncontained landfills produce wider plumes (Christensen *et al.*, 2001).

1.5 WASTE DEGRADATION

Landfills release a wide range of chemicals resulting from waste disposal and subsequent degradation in the form of leachate, gas and particulate matter (PM), although the latter is only released from active landfills (Defra, 2004). The gas is collected, often as a condensate due to the moisture inherent within MSW landfills, and pipes on-site collect leachate. Although most sites utilise water-sprays or bowsers to mitigate levels of PM, this is usually restricted to roadways in use and there are no regulations pertaining to PM collection on landfills. Subsequently, PM is released into the ambient air (DoE, 1986; Rushton, 2003; Macleod *et al.*, 2006; Koshy *et al.*, 2009).

1.5.1 LANDFILL LEACHATE

MSW landfill chemistry is complex due to the heterogeneous nature of the waste. Waste has many characteristics that affect its decomposition; solubility, leachability, biodegradability, combustability, volatility, chemical composition, object size and the heterogeneity of the waste (Kurniawan *et al.*, 2006; McBean *et al.*, 2007). Leachate is formed from the water content of the waste at the time of burial, as well as moisture infiltration from rain, snow and surface channels. As this water (generated during degradation) percolates through the system, it picks up suspended PM, dissolved organic and inorganic chemicals, moving them through the waste to react with other species. There are generally

5 stages of organic decomposition of waste (Figure 1.6), which begins with (I) a short aerobic degradation, followed by significantly longer anaerobic (II) fermentation and hydrolysis, (III) acetogenesis and (IV) methanogenesis, before conditions return to (V) the aerobic state (Kjeldsen *et al.*, 2002; Williams, 2002; Östman *et al.*, 2006).

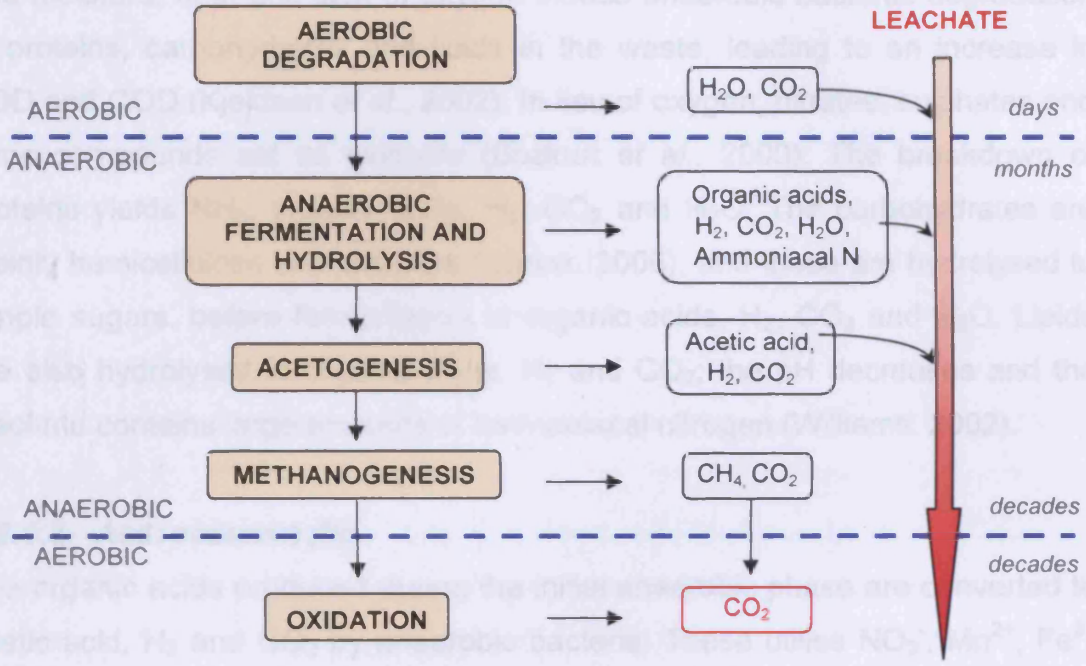

Figure 1.6: *The 5 major stages of bioreactive waste degradation in landfills and their products which form leachate or are released as CO_2.*

1.5.1.1 *Aerobic Degradation (I)*

Once buried, aerobic microorganisms metabolise organic components and quickly deplete the available oxygen in the waste within a few days (Kjeldsen *et al.*, 2002). This stage mainly produces CO_2 and H_2O, and an increase in the temperature to 70-90°C (Williams, 2002). The leachate formed at this stage is mainly from the mechanical compaction process and hence exhibits low values of Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD). These are both measures of oxygen-depleting capacity of compounds present in the waste water. BOD measures microbiological consumption of oxygen due to the levels of biodegradable organic matter present in the leachate, whilst COD is a measure of the total amount of oxidisable matter, including the non-

biodegradable components. The lowering in COD can be achieved by leachate treatment facilities before the effluent can be released into foul sewer systems; depleted COD is an indication of desirable leachate stabilisation (Gotavjn *et al.*, 2009).

1.5.1.2 Anaerobic Hydrolysis and Fermentation (II)

The moisture, heat and lack of oxygen induce anaerobic bacterial degradation of proteins, carbohydrates and lipids in the waste, leading to an increase in BOD and COD (Kjeldsen *et al.*, 2002). In lieu of oxygen, nitrates, sulphates and ferric compounds act as oxidants (Bozkurt *et al.*, 2000). The breakdown of proteins yields NH_3, organic acids, H_2, CO_2 and H_2O. The carbohydrates are mainly hemicellulose and cellulose (Barlaz, 2006), and these are hydrolysed to simple sugars, before fermentation to organic acids, H_2, CO_2 and H_2O. Lipids are also hydrolysed to organic acids, H_2 and CO_2; the pH decreases and the leachate contains large amounts of ammoniacal nitrogen (Williams, 2002).

1.5.1.3 Acetogenesis (III)

The organic acids produced during the initial anaerobic phase are converted to acetic acid, H_2 and CO_2 by anaerobic bacteria. These utilise NO_3^-, Mn^{2+}, Fe^{3+} and SO_4^{2-} as oxidants in the absence of O_2 (Christensen *et al.*, 2001). The generation of organic acids – most notably acetic acid - leads to an increase in acidity, in some cases to pH 4, as well as a peak in BOD and COD. The elevated acidity results in the increased mobility of metal ions in the leachate. These can proceed to react with other soluble ions (e.g. PO_4^{2-}, Cl^-, NH_4^+) and can relocate (Bozkurt *et al.*, 2000).

1.5.1.4 Methanogenesis (IV)

During this final anaerobic phase, the two main kinds of methanogenic microbes are promoted by the products of earlier acid-generating phases. The acetic acid produced in earlier phases is converted to CH_4 and CO_2 by *mesophilic* microbes at 30 – 35°C and *thermophilic* species at 35 – 65°C (Tuomela *et al.*, 2000). The concentration of organic acids is gradually depleted and the pH rises to neutral levels, reducing the mobility of free metal ions in the

leachate. Sulphate-reducing bacteria produce H_2S throughout the anaerobic phase by consuming H_2. The majority of H_2S remains aqueous and can react with the solubilised metal ions, causing their precipitation as metal sulphides (Suna Erses and Onay, 2003). The system becomes reducing, and the redox potential falls to below 0mV, thereby increasing the redox capacity of the system. The redox capacity is a measure of the amount of oxygen that the system can react with, meaning the leachate remains highly reducing during the anaerobic phase (Bozkurt *et al.*, 2000). Methanogenesis is the most stable and durable stage in landfill degradation, beginning within 6 months of waste deposition, and is expected to last at least 30 years (Williams, 2002). Since significant amounts of CH_4 are generated, it can be the most significant period in energy recovery from LFG.

1.5.1.5 Aerobic Oxidation (V)

This phase has not been studied in modern landfills, as it is predicted that it may take up to 100 years for a landfill to reach this stage of evolution, and most monitored landfills are less than 40 years old (Bozkurt *et al.*, 2000). This postulated aerobic oxidation phase is expected to happen decades after methane generation has ceased, and after the easily degradable organics have been depleted. Also called the humic phase, this long-term stage in the lifetime of a landfill depends on the entry of O_2, which may be retarded by a good clay cover and the presence of liquid within the waste mass (Kjeldsen *et al.*, 2002).

Although certain assumptions about leachate composition can be made if the waste stream is well-characterised, the end-products released are highly dependent upon the reactions which occur within the waste mass. In practice, leachates from waste of varying ages are mixed in the drainage system before release from landfills. In general, leachate will contain ammonia and organic matter from biodegradation, and will also be highly reducing (Östman *et al.*, 2006). Typical UK leachate parameters are provided in Table 1.2.

The most common metals found in leachate include Fe and Mn (DoE, 1995). Iron and Mn are reduced from their solid, oxidised, Fe^{3+} and Mn^{4+} oxides and hydroxides, to form soluble Fe^{2+} and Mn^{2+} ions. These cations can move by

Parameter	Concentration range mg/L	
	Acetogenic	Methanogenic
pH	5.12 – 7.8	6.8 – 8.2
Electrical conductivity µS/cm	5800 - 52000	5990 - 19300
TOC	1010 - 29000	184 - 2270
BOD_5	2000 - 68000	97 - 1770
COD	2740 - 152000	622 - 8000
Inorganic macrocomponents		
Nitrogen (Ammonia)	194 - 3610	283 - 2040
Chloride	659 - 4670	570 - 4710
Calcium	270 – 6240	23 – 501
Sulphate	<5 – 1560	<5 – 322
Phosphate	0.6 – 22.6	0.3 – 18.4
Sodium	474 - 2400	474 - 3650
Potassium	350 - 3100	100 - 1580
Magnesium	25 - 820	40 - 1580
Iron	48 – 2300	1.6 – 160
Manganese	1 - 164	0.04 – 3.59
Extractable metals		
Arsenic	<0.001 – 0.148	<0.001 – 0.485
Cadmium	<0.01 –0. 10	<0.01 – 0.08
Chromium	0.03 – 0.30	<0.03 – 0.56
Copper	0.02 – 1.10	<0.02 – 0.62
Nickel	<0.03 – 1.87	<0.03 – 0.60
Lead	<0.04 – 0.65	<0.04 – 1.9
Mercury	<0.0001 – 0.0015	<0.0001 – 0.0008
Zinc	0.09 -140	0.03 -6.7
Trace organic components	1010 - 29000	1010 - 29000

Table 1.2: *Composition of acetogenic and methanogenic leachates from 29 large and relatively dry landfills. Adapted from WMP26B (DoE, 1995).* [a]*All units are in mg/L except for pH and conductivity.*

leaching through voids in waste before being oxidised and precipitating out as their sulphides and organic complexes during landfill degradation, hence their concentrations fall during methanogenesis (Christensen *et al.*, 2001; Suna Erses and Onay, 2003). Ferric iron hydroxide is a good adsorbant for other metals, therefore leaching of Fe^{2+} is usually accompanied by other metals (Bozkurt *et al.*, 2000). Potassium, Na, Ca and Mg are not subject to these redox reactions, but they do take part in cation exchange processes, enabling their translocation within the waste (Figure 1.7).

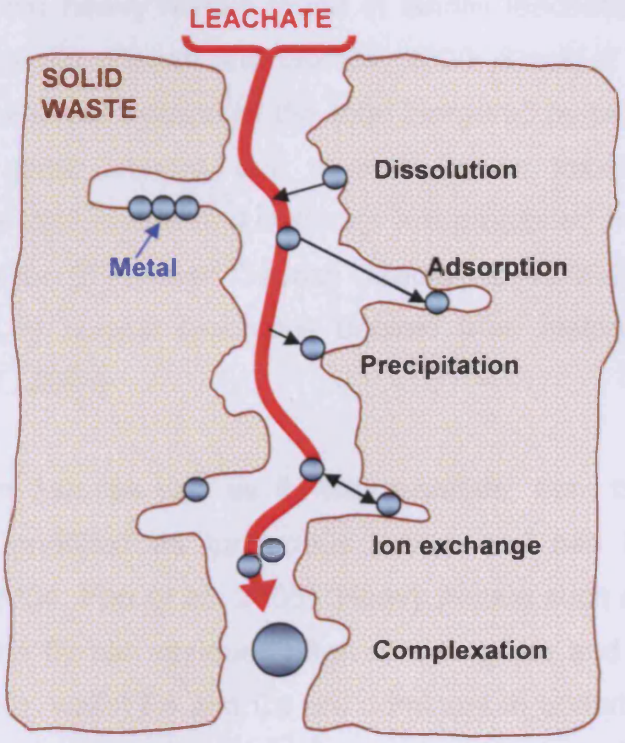

Figure 1.7: *Leachate migration through solid landfilled waste illustrating the major metal attenuation (adsorption, precipitation, ion exchange) and mobilisation (dissolution, ion exchange, complexation) processes.*

The low pH and high levels of dissolved organic matter during acetogenesis will increase the mobility of most metals. Metals will bind with the wide array of ligands available – carbonate, chloride, hydroxides, as well as humic acids (Baun and Christensen, 2004). Humic acids are large molecular weight organic complexes produced after methanogenesis of the waste, and may be either soluble or insoluble. Therefore, metal ions, which bond with humic acids, may

be solubilised into leachate as organic complexes or immobilised as colloidal particles (Suna Erses and Onay, 2003). The rise from pH 4 to pH 7-8 seen during methanogenesis will retain metals within the waste mass. Significant precipitation and sorption onto solid waste particulates occurs, as dissolved organic matter that would normally have complexed metal ions, are degraded during methanogenesis and become unavailable for metal binding (Bozkurt *et al.*, 2000). The diameter of leachate metal species may range from <0.001µm, to large organic complexes approaching 10µm (Baun and Christensen, 2004).

Commonly occurring heavy metals found in landfill leachates include Cd, Cr, Cu, Pb, Hg, Ni and Zn (Ceçen and Gürsoy, 2000; Slack *et al.*, 2005). Heavy metals represent a small fraction of the total inorganic by-products of landfills, but they are of great interest, due to their known toxicity (Järup, 2003). Although electrical appliances and batteries are prohibited from MSW landfills by modern legislation (European Council Directive 2002/96/EC), heavy metals are still detected in current leachates derived from historical waste tipping (Christensen *et al.*, 2001).

Incinerator bottom ash, as well as fly ash residues, from cleaning stacks of incinerators are landfilled as hazardous waste and can leach out metals (Radetski *et al.*, 2004; Fan *et al.*, 2006). Heavy metals such as Hg and Cd are concentrated in the fly ash residues after condensation and nucleation of PM (Lighty *et al.*, 2000), whilst Fe and Cu are dominant in bottom ash (Jung *et al.*, 2004). Since older landfills did not differentiate between inert and hazardous sources when accepting waste, ash-sourced metals can occur in current leachates from discontinued old landfills (Östman *et al.*, 2006).

1.5.2 LANDFILL PARTICULATE MATTER

The issue of air pollution has been historically well-documented in the UK, dating from the report of John Evelyn to Queen Elizabeth I in 1661, which recommended the relocation of factories away from London, for control of excessive airborne soot (Brimblecombe, 1988). Industrial and domestic coal-burning in the early 20th century culminated in a handful of events which

dramatically illustrated the impact of air pollution upon human health. These were the (i) 5-day fog in Meuse Valley, Belgium 1930, which resulted in 63 fatalities, (ii) Donora winter fog, Pennsylvania 1948, which caused 20 deaths and 7000 cases of morbidity, (iii) London smog, 1952, which resulted in over 4000 extra deaths and (iv) the 1985 – 1988 Geneva Steel mill study in Utah (Pope and Dockery, 2006). Substantial data now exists to indicate that particulate air pollution is associated with adverse human heath effects such as respiratory and cardiovascular conditions (Dockery *et al.*, 1993; Brunekreef and Holgate, 2002).

Inhalable airborne PM less than 10μm in diameter can be divided into three fractions (EPAQS, 2001) and all may be formed on a landfill site:

1. Coarse: Particles have an aerodynamic diameter between 10 and 2.5μm. Aerodynamic diameter is defined as the physical diameter of a smooth sphere of density $1g/cm^3$, which has the same settling velocity, in still air, as the particle of interest (Phalen *et al.*, 2006). Physically abrasive and mechanical processes, such as the erosion of crustal surfaces, sea spray, natural bioaerosols and vehicle emissions largely form these particles (Wilson *et al.*, 2002). These PM are suspended and dispersed by wind, but usually deposit by sedimentation within a few hours.

2. Fine: Particles have an aerodynamic diameter between 2.5 and 0.1μm. The majority of these particles are formed as a condensation product of chemical vapours and primary particle aggregations that coagulate into a slow-settling fraction, remaining airborne for longer than coarse PM (Wilson *et al.*, 2002). This fraction also consists of PM from the lower tail of the coarse-mode PM, meaning fine PM exhibits complex chemistry.

3. Ultrafine: Particles with an aerodynamic diameter of less than 0.1μm. The most significant source of these particles is the internal combustion engine, in particular, diesel exhaust particles that rapidly aggregate into spherules. These become aggregated into the fine mode (BéruBé *et al.*, 1999). In this way,

chemical contaminants can be adsorbed onto the surface of particulate matter and transported further afield (de Kok *et al.*, 2006).

The particulate matter load in the atmosphere can generally be classified as either primary or secondary particles, based on their chemical composition (AQEG, 2005). Primary PM are formed at the emission source, predominantly originating from either stationary (power stations, domiciles) or mobile (aircraft, vehicular) combustion. In UK urban air, this is mostly in the form of diesel exhaust particles (DEP) from incomplete internal engine combustion, which produces soot and inorganic PM and a range of adsorbed compounds (Harrison and Yin, 2000). Although gasoline-fuelled vehicles also generate soot mass, the particle numbers tend to be several orders of magnitude less than diesel engines: approximately 15-30mg/m^3 DEP corresponds to 10^{15} particles, whilst 0.1mg/m^3 gasoline-derived PM corresponds to 10^{11} particles (Lighty *et al.*, 2000). Primary PM may also include coarse fraction crustal particulates that can make a significant contribution to pollution particulate mass, albeit a minor proportion of particle numbers (Harrison and Yin, 2000; AQEG, 2005). Secondary PM refer to those formed by atmospheric gaseous reactions, including gas-to-particle transitions (predominantly ammonium sulphates and nitrates), in addition to hydrocarbons, which represent the more soluble carbonaceous matter; these tend to be highly soluble (AQEG, 2005).

The bulk chemical composition of urban PM around the world tends to exhibit variable proportions of the same components (Viana *et al.*, 2008):

- Sulphate: long-range transport-indicator from oxidation of SO_2 in the atmosphere
- Nitrate: from oxidation of atmospheric NO_x, faster oxidation rates than sulphate formation
- Chloride: released by sea-spray and road de-icing activities
- Minerals: comprised of either major (Al, Si, Ca, Mg, K) or trace elements (transition metals)
- Ammonium: formed by the neutralising reaction of atmospheric agricultural ammonia, with sulphate and nitrate

- Organic: combustion-derived particles, atmospheric photochemical reactions that form volatile organic compounds and biogenic sources (Harrison *et al.*, 2004; Putaud *et al.*, 2004; AQEG, 2005).

The potential toxicity and adverse health effects of PM is dependent upon the interaction with the respiratory system. As the adult human may inhale 16-20m^3 of air daily (Baeza-Squiban *et al.*, 1999), exposing the respiratory tract to a myriad of xenobiotics, the body has developed efficient clearance mechanisms, including the sneeze and cough reflexes. The extent to which PM deposit and are retained in the airways is a function of the airflow, penetration and clearance mechanisms, as summarised in Table 1.3 and Figure 1.8 (Squadrito *et al.*, 2001; Mohanraj and Azeez, 2004).

ESD	Main Deposition Site	Deposition	Clearance
> 10μm	Nasal, pharangeal passages and extra-thoracic regions.	Impactiion	Mucociliary transport
5-8μm	Tracheobronchial tree	Interception, impaction	Mucociliary transport
0.5-5μm	Respiratory bronchioles and alveoli	Diffusion, Brownian motion	Slow clearance; active transport by macrophages to cilia
< 0.5μm	Alveoli	Exhaled	

Table 1.3: *Penetration, deposition and clearance of inhaled particles according to their aerodynamic equivalent spherical diameter (ESD). Adapted from Squadrito et al., 2001).*

Large PM (> 10μm) inhaled through the nose or mouth, and are filtered out in the upper respiratory tract by deposition onto non-ciliated sites in the nose, or impaction on mucus surfaces of the extra-thoracic region (Eccles, 2006). Trapped PM are moved to the oesophagus for ingestion; PM < 10μm proceed into the thoracic region, where the air is humidified. As PM enters the ciliated tracheobronchial tree, the increasing number of narrowing bifurcations leads to a fall in air velocity, and PM 1-8μm are captured on the mucus, especially at

focal points (Lippmann and Ito, 2000). These thoracic-deposited PM are moved upwards, out of the lungs via the mucociliary escalator for coughing and ingestion. The smallest PM enter the alveolar zone of the distal respiratory tract, distinguished by the immense surface area and extensive pulmonary vasculature. These are non-ciliated areas, patrolled by alveolar macrophages which phagocytose foreign PM for transport away from alveoli, to the ciliated airways, or into the lymphatic system (Mohanraj and Azeez, 2004).

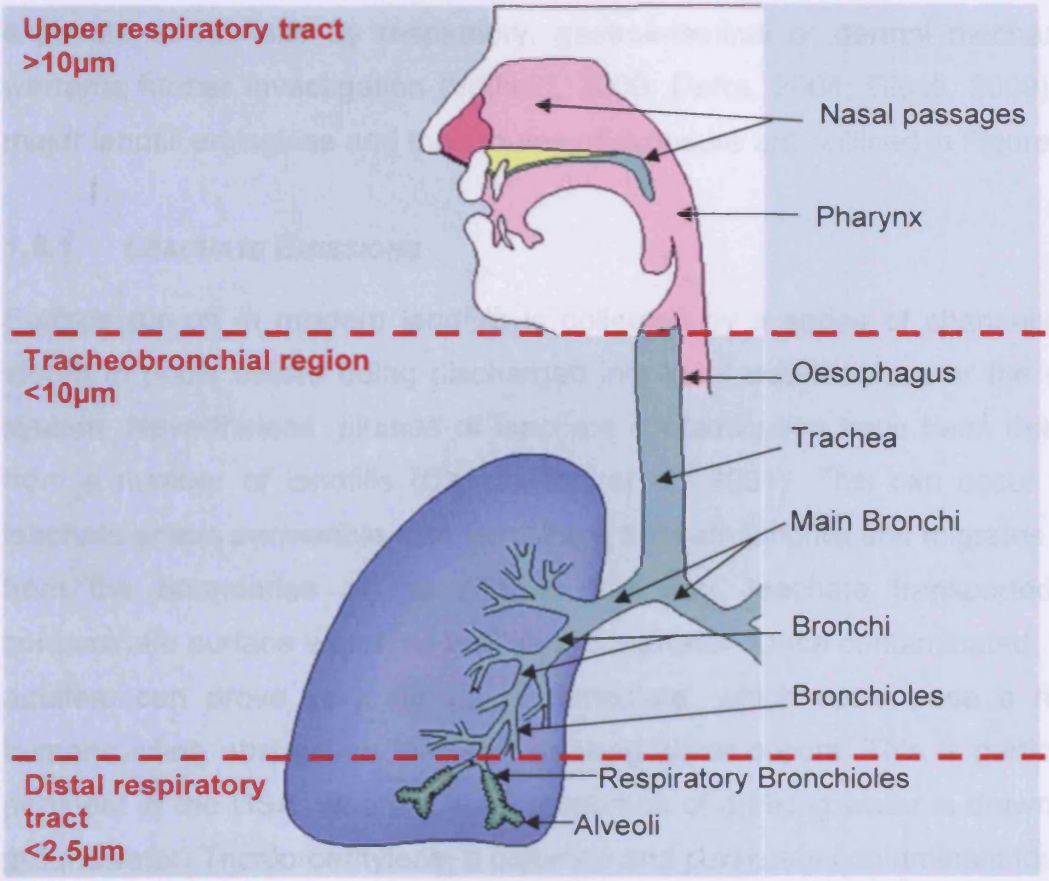

Figure 1.8: *Anatomy of the respiratory system and PM deposition sites.*

Current data suggests greater biological effects from fine and ultrafine PM may be due to several factors, including the hypothesis that these PM may be deposited in the alveoli in sufficient numbers to saturate the clearance capacity (Lighty *et al.*, 2000). Indeed, it was reported by Linn *et al.*, (2000) that persistent, insoluble PM which deposit in alveoli, have been retained for weeks, months, or even years. The greater surface-area to mass ratio would also enhance solubility of fugitive adsorbed chemicals that may be translocated to

extrapulmonary organs via the systemic circulation (Nemmar *et al.*, 2004; Mills *et al.*, 2009).

1.6 LANDFILLS AND HEALTH EFFECTS

With 80% of the UK population living within 2km of a landfill (Elliot *et al.*, 2001), there is great interest in the potential adverse health risks posed by such sites and their emissions, i.e. leachate, gases and PM. Therefore, the potential exposure to humans by respiratory, gastrointestinal or dermal mechanisms warrants further investigation (Vrijheid, 2000; Defra, 2004; Giusti, 2009). The major landfill emissions and their routes of exposure are outlined in Figure 1.9.

1.6.1 LEACHATE EMISSIONS

Surface run-off in modern landfills is collected by a series of channels, and stored in pools before being discharged into local watercourses or the sewer system. Nevertheless, plumes of leachate contamination have been detected from a number of landfills (Christensen *et al.*, 2001). This can occur when leachate enters permeable rock formations beneath landfills and migrates away from the boundaries of the site. In this way, leachate transported can contaminate surface water, as well as groundwater. Once contaminated, these aquifers can prove very difficult to remediate, which could pose a risk to humans when abstraction to obtain drinking water occurs. This is particularly pertinent in the USA, where a large proportion of drinking water is drawn from groundwater. Trichloroethylene, a common and persistent contaminant found in groundwater has been linked to liver and lung cancer (Fisher, 1993). In the UK, the potential for occupational dermal exposure may pose a greater risk than that of ingestion by the public (Defra, 2004).

1.6.2 LANDFILL PARTICULATE MATTER EMISSIONS

Airborne landfill PM is a complex combination of multiple components such as dusts with adsorbed organic compounds, diesel exhaust particle (DEP) fumes and bioaerosols (Redfearn and Roberts, 2002). Exposure to the human population can occur via respiratory, dermal and ingestion routes and is

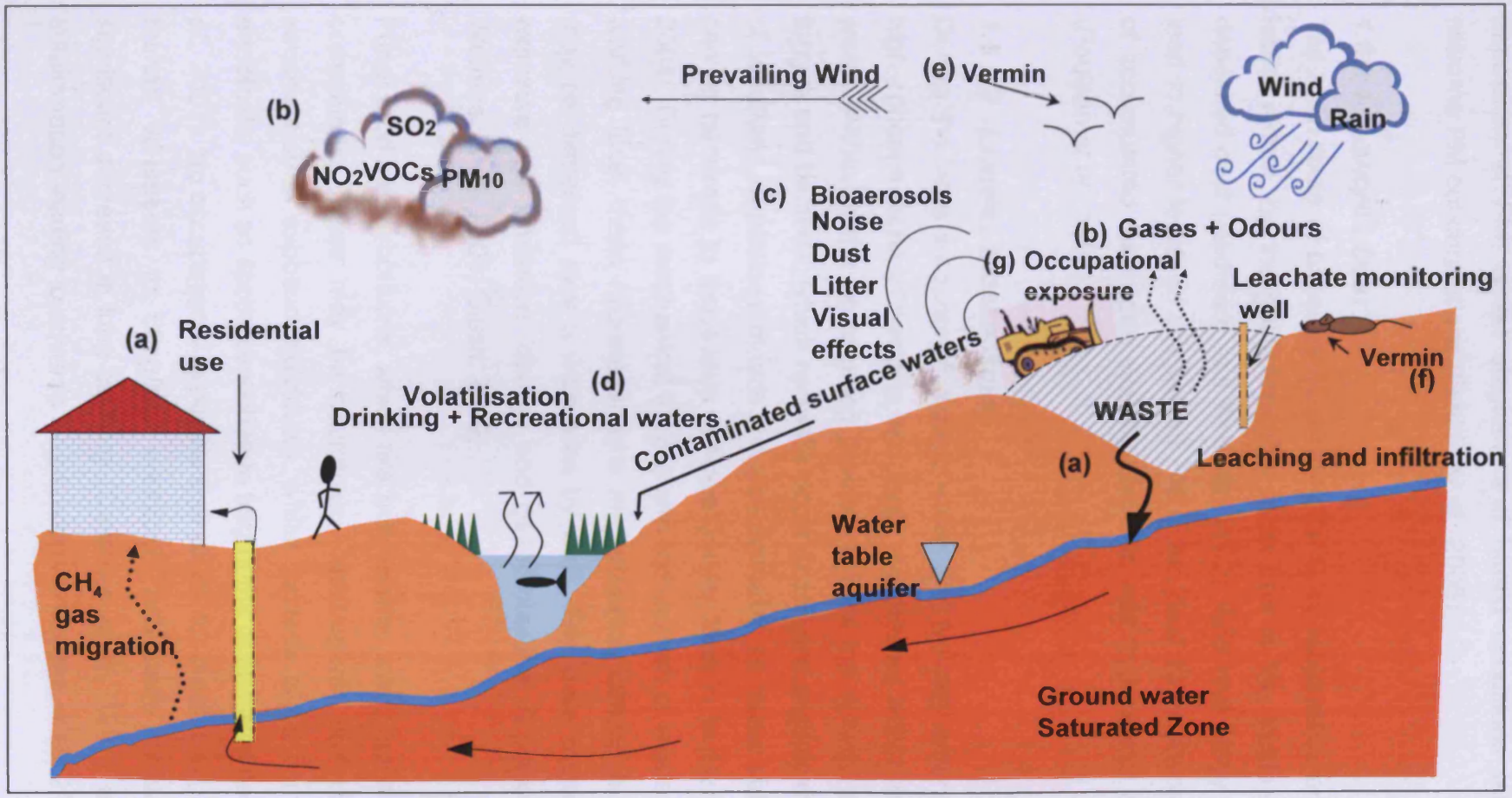

Figure 1.9: *Routes of pollutant exposure from landfills, featuring landfill leachate, gas and PM.* ***(a)*** *Leachate may leak, contaminating groundwater.* ***(b)*** *Volatile landfill gases and odours dispersed by wind, cause public concern.* ***(c)*** *Landfill PM emissions are usually accompanied by noise and visual effects.* ***(d)*** *Harmful substances may be released into recreational waters from leachate and contaminated surface waters. Vermin also persist around landfills and predominantly include* ***(e)*** *seagulls and* ***(f)*** *rodents, both of which contribute to dispersal of waste.* ***(g)*** *Occupational exposure to landfill workers and waste handlers.*

sensitive to meteorological conditions. High levels of precipitation leads to wet deposition of PM, whilst stagnant and humid conditions tend to increase airborne PM concentrations (Elminir *et al.*, 2005).

1.6.2.1 LANDFILL DUST

The main route of exposure to landfill dust is by respiration, and possibly to a lesser extent, by the gastrointestinal route due to the incidental ingestion of deposited dust (Redfearn and Roberts, 2002). Arid and windy conditions may lead to higher levels of suspended PM in the local atmosphere. Re-dispersion of accumulated dust upon clothing can also add to personal exposure to PM (Poulsen *et al.*, 1995).

1.6.2.2 LANDFILL BIOAEROSOLS

Due to the large amounts of organic waste that typically enters MSW landfills, high concentrations of bacteria and fungi are present within the decomposing waste. Although the composting process utilises the activity of microbes, i.e. fungus and bacteria, which naturally occur in soil and organic matter, the scale of landfilling operations means the concentration of these airborne microbes can be hundreds to thousands of times greater than in ambient air (Lis *et al.*, 2004). During the mechanical turning and compaction of waste at the exposed working face, these microorganisms are released, forming a bioaerosol that may be dispersed over a wide area by wind. The main routes of bioaerosol exposure are inhalation, dermal and gastrointestinal (Poulsen *et al.*, 1995; Wouters, *et al.*, 2006; Giusti, 2009).

Fungi produce mycotoxins, which are non-volatile, toxic, low molecular-weight compounds. These may be neurotoxic, carcinogenic and cause allergenic sensitisation in exposed individuals, whilst bacteria are a common source of endotoxin, such as lipopolysaccharide fragments of bacterial cell wall (Sykes *et al.*, 2007). No occupational exposure limit exists for bioaerosol, but exposure of human volunteers to 50ng/mg^3 endotoxin has been shown to result in significant decrease in lung capacity (Castellan *et al.*, 1987), and non-specific inflammation leading to adverse lung function (Wouters *et al.*, 2006).

On-site composting programmes at MSW landfills are likely to increase as legislation pushes for a reduction in bioactive waste buried in landfills (European Council Directive 1999/31/EC), leading to an increased risk in bioaerosol exposure. The practice of dilute leachates or liquid waste irrigation onto open land can also give rise to bioaerosols although here, dermal exposure is the main exposure pathway (Gray *et al.*, 2005).

1.6.3 Occupational Targets

A large workforce is required for the collection and disposal of MSW. Dependant on the operating conditions, they can be exposed to any of the landfill emissions of leachates, LFG and PM. Previous studies have found site offices near dustcart weigh-bridges contain elevated concentrations of bacteria and fungi (Lis *et al.*, 2004). Operators of enclosed site vehicles may be exposed to excessive concentrations of PM regardless of the presence of air filters in the cabin compartment (Mozzon *et al.*, 1987), whilst those lacking air conditioners may be operated with the windows open and thus be under direct exposure to PM. In less regulated landfilling operations, such as those found in developing nations, workers tend to suffer from a range of general health problems, including respiratory, gastrointestinal and neurological symptoms (Ray *et al.*, 2005).

1.6.4 Community Targets

Overall, the adverse effects perceived by local residents have tended to be due to the myriad of undesirable factors that accompany landfills. These include the risk of explosive CH_4 levels, dustcart traffic, visual intrusion upon the landscape and high levels of nuisance dust, pests and unpleasant odours (Figure 1.19). However, people residing near landfills may also be exposed to chemicals released in the air, soil or water (Vrijheid, 2000). Within the UK, contaminated recreational waters may be used for angling and water sports, leading to acute incidental exposure (ATSDR, 2003). Although the presence of groundwater abstraction boreholes proximal to landfill sites is less common in the UK than the USA, this can nevertheless result in chronic community exposure (ATSDR, 2003). Unpleasant odours reported near landfills (especially mixed-waste sites)

are primarily due to gaseous compounds such as volatile fatty acids, and excessive H_2S may arise from co-disposed gypsum (Parker *et al.*, 2002; de la Rosa *et al.*, 2006; Colledge and Wilder, 2008). Moreover, exposure to ammonia, and the many toxic VOCs such as benzene and limonene, which escape in LFG, can be a concern (Parker, 2002; Hamoda, 2006). The intensity and properties of LFG odour are dependent on the waste composition and its stage of decomposition within the landfill. The extent to which airborne pollutants are diluted by ambient air depends on the local meteorological conditions – rain, wind, temperature and pressure. Rainfall, humidity, stable atmospheric temperatures and pressure hinder LFG migration, whereas windy conditions increase LFG dispersion and dilution (El-Fadel *et al.*, 1997; Boltze *et al.*, 1997).

Many of the trace organic components in LFG are toxic, yet LFG has historically been considered as being more of a nuisance than a hazard to human health (El-Fadel *et al.*, 1997). It has been shown that the composition of indoor PM_{10} is closely determined by outdoor sources (Jones, 1999; BéruBé *et al.*, 2004), and that buildings can act as concentrators of particulates, increasing the risk to residents. Thus, VOCs and pollutants such as DEP sourced from the increased heavy road traffic passing near residences may exacerbate pre-existing respiratory and cardiovascular conditions (Pope *et al.*, 1999; Anderson *et al.*, 2001; Castranova *et al.*, 2001; Cacciola *et al.*, 2002).

1.7 ANTIOXIDANTS AND METAL CHELATION

A growing body of evidence supports the catalytic role of transition metals (TM) in the deterioration of biological molecules, with the toxicity attributed to the redox capacity of these cations. A plethora of diseases in which reactive oxygen species (ROS) and oxidative stress are implicated have been described in the literature. These include inflammation, cancer, cardiovascular and neurodegenerative diseases (Halliwell, 1996; Dröge, 2002; Klaunig and Kamendulis, 2004). The basic mechanisms involved in the generation of ROS from metals, share common routes. The Fenton reaction, first reported in 1894,

described the reaction between Fe^{2+} and H_2O_2, which forms the highly reactive hydroxyl radical ($^{\bullet}OH$), and can occur under either *in vitro* or *in vivo* conditions (Imlay *et al.*, 1988; Valko *et al.*, 2005). Halliwell (1987), described a radical as "any species capable of independent existence that contains one or more unpaired electrons". ROS include oxygen radicals and related species such as H_2O_2, O_3, which themselves do not possess unpaired electrons, but are often involved in the generation of radicals. The Fenton reaction is now known to be applicable to multivalent TM, including Cu, V, Cr and Co (Valko *et al.*, 2007). The oxidised TM cation can react with the superoxide anion ($O_2^{\bullet-}$), converting the metal back to the reduced state. The sum of these two reactions is known as the Haber-Weiss reaction, which would normally only progress at a slow rate; however in this case, is catalysed by the presence of Fe^{3+} (Equations I – III). The $^{\bullet}OH$ moiety is the neutral form of the hydroxide ion, and is the most biologically reactive ROS known. It has an extremely short *in vivo* half-life (10^{-9} seconds; Pastor *et al.*, 2000), thereby implicating its rapid reaction with nearby molecules (Valko *et al.*, 2007). In this oxidant scenario, H_2O_2 may be provided by normal endogenous metabolism – including $O_2^{\bullet-}$ dismutation by superoxide dismutase - since it is an intracellular signaling molecule. Alternatively, $O_2^{\bullet-}$ can be reduced non-enzymatically to H_2O_2 by reaction with other free radicals and reducing equivalents (Squadrito *et al.*, 2001):

(I)	$Fe^{2+} + H_2O_2$	$\longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$
(II)	$Fe^{3+} + O_2^{\bullet-}$	$\longrightarrow Fe^{2+} + O_2$
(III) Net reaction	$H_2O_2 + O_2^{\bullet-}$	$\longrightarrow {}^{\bullet}OH + O_2 + OH^-$

Efficient enzymatic and non-enzymatic antioxidant defences exist in cellular systems; nonetheless, these may reach saturation. The biological targets most susceptible to oxidation from endogenous and exogenous ROS sources can be categorised as:

- DNA damage – single and double-strand breaks, intra- and inter-stand links, protein-nucleotide cross-links
- Lipid peroxidation – sustained generation of free radicals, alteration of cellular membrane fluidity

- Protein modification – sulfhydryl groups oxidised to disulfides, loss of function
- Carbohydrates – oxidation of cell surface receptors, effects upon ligand-binding (Nordberg and Arnér, 2001; O'Brien *et al.*, 2005).

Although nuclear DNA is well-protected by histones and the nuclear membrane, the presence of several nucleophilic sites on the molecule means that DNA is susceptible to attack from electrophilic ROS at double-bonds, O and N atoms. Since H_2O_2 readily diffuses across membranes, it can migrate to the nuclear DNA, and take part in Fenton chemistry, thereby generating $^{\bullet}OH$ which can either modify bases, disrupting intramolecular hydrogen-bonding or form sugar-radicals which result in fracturing of the deoxyribose backbone (Klaunig *et al.*, 1998; Valko *et al.*, 2007). PM have been shown to cause increased DNA oxidative damage in human airway epithelial cells, which was associated with the amount of water-soluble metals contained on these particles (Prahalad *et al.*, 2001).

The attack by free radical ROS upon cytosolic, nuclear or membrane polyunsaturated fatty acids in lipids, which are rich double bonds, can initiate a self-propagating radical chain reaction. If unquenched, this may lead to severe cellular disruption, causing losses in membrane fluidity, receptor and transporter function, cell signalling and eventual cell death. Lipid peroxidation is a precursor to the formation of atherosclerotic plaques, which are implicated in cardiovascular disease (Araujo *et al.*, 2008). Since lipid radicals can diffuse through membranes, they can propagate the oxidation of other macromolecules, posing an additional risk to the redox balance.

ROS can also attack any amino acid, whether it is bound as a polypeptide(s) or free; cysteine and methionine residues are particularly susceptible to oxidation. These modified proteins form peroxides and protein radicals which terminate in inter-molecular cross-links, altered tertiary structures and protein fragmentation (Stadtman *et al.*, 2001). A recent study by Sørensen *et al.*, (2003), reported a significant association between combustion-derived PM exposure and oxidised

plasma proteins. The oxidation of such proteins and lipids contributes to the onset of artherosclerotic plaques and cardiovascular disease. In addition, oxidative damage to cellular proteins may lead to altered signalling and enzyme function, and has been associated with senescence (Finkel, 2000; Dröge, 2002).

1.7.1 Respiratory Tract Lining Fluid

Halliwell and Gutteridge (1995) defined an antioxidant as "any substance that when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate." Since it is the first line of defence against inhaled xenobiotics which may be oxidative, the respiratory tract mucosal surfaces are lined with a rich milieu of antioxidants which vary in concentration according to the anatomical branching (Putman *et al.*, 1997; Cross *et al.*, 1998, 2002). The respiratory tract lining fluid (RTLF) exists in two phases i.e. aqueous and gel layers. Proximal RTLF tends to be thicker, due to the presence of mucous glycoproteins contributing to the gel phase, whilst distal alveolar regions are more aqueous and contain higher concentrations of low molecular weight antioxidants (Kelly *et al.*, 2003). A validated model of RTLF has been used by previous investigators as a surrogate epithelial lining fluid (SELF), containing the primary non-enzymatic, water-soluble antioxidants: Uric Acid (UA), Ascorbic Acid (AA) and reduced Glutathione (GSH) (Zielinski *et al.*, 1999; Greenwell *et al.*, 2002a; Mudway *et al.*, 2004). These ROS-scavenging molecules play a vital role in protecting the underlying tissue from cellular damage (Figure 1.10).

Uric acid is the end-product of purine metabolism and known as a highly efficient hydroxyl scavenger (Figure 1.11.a). It is a major proximal airway antioxidant, ranging from ~400μM in nasal passages, to ~150μM in distal RTLF (Mudway and Kelly, 2000). The reduction of ROS by UA leads to generation of a relatively stable UA radical, which is regenerated back to UA by ascorbic acid (AA). Due to its higher concentration at the upper airway and predilection for ROS (e.g. O_3), UA may act as a "scrubber" for inhaled air, thereby protecting both upper, and more susceptible lower airways (Cross *et al.*, 2002).

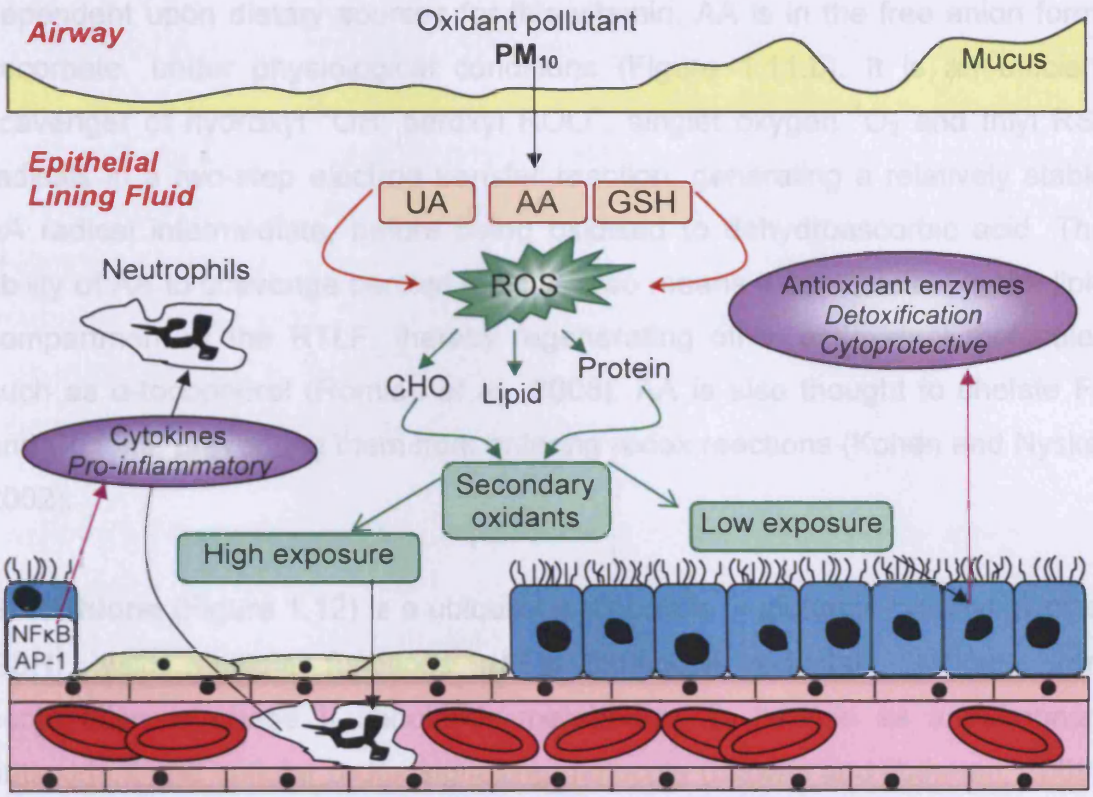

Figure 1.10: *Simplified mechanism of oxidative stress and biochemical response in the airway. If the antioxidant defences provided by (but not limited to) UA (uric acid), AA (ascorbic acid) and GSH (reduced glutathione) are overwhelmed, the excess ROS can oxidise carbohydrates, lipids, and protein, resulting in altered epithelium. The activation of redox-sensitive transcription factors (such as NFκB and AP-1) will propagate a cellular inflammatory response.*

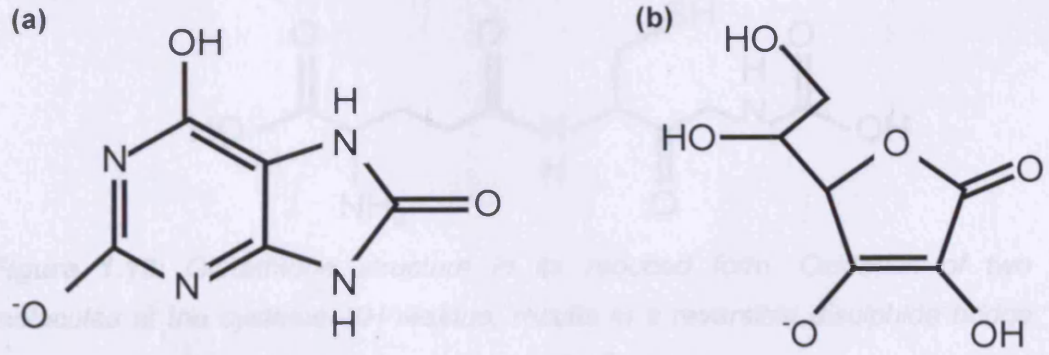

Figure 1.11: *RTLF components at physiological pH.* ***(a)*** *Uric acid is present as urate* ***(b)*** *Ascorbic acid is present as ascorbate anion.*

Ascorbic acid is not produced endogenously, meaning humans are completely dependent upon dietary sources for this vitamin. AA is in the free anion form, ascorbate, under physiological conditions (Figure 1.11.b). It is an efficient scavenger of hydroxyl $^{\bullet}OH$, peroxyl $ROO^{\bullet}$, singlet oxygen $^1O_2$ and thiyl $RS^{\bullet}$ radicals in a two-step electron transfer reaction, generating a relatively stable AA radical intermediate, before being oxidised to dehydroascorbic acid. The ability of AA to scavenge peroxyl radicals also means it can function in the lipid compartment of the RTLF, thereby regenerating other antioxidant molecules such as α-tocopherol (Romieu *et al.*, 2008). AA is also thought to chelate Fe and Cu ions, preventing them from entering redox reactions (Kohen and Nyska, 2002).

Glutathione (Figure 1.12) is a ubiquitous tripeptide (γ-glutamyl-cyteinyl-glycine; GSH), with multiple functions as a biological reductant, ranging from conjugation in phase II xenobiotic metabolism, to its role as a pulmonary antioxidant and cellular redox signalling molecule (Biswas and Rahman, 2009). GSH, by virtue of its reactive thiol residue, can chelate metal ions and reacts directly with $^{\bullet}OH$, $ROO^{\bullet}$, alkoxyl $RO^{\bullet}$ and $O_2^{\bullet-}$ radicals. In doing so, GSH itself becomes a thiyl ($GS^{\bullet}$) radical, which can be regenerated intra-cellularly, whilst the GSH-conjugates are transported out of the cell (Kohen and Nyska, 2002). GSH has been found at up to 400μM in normal human RTLF, albeit in the distal alveolar regions, whilst upper airways present much more dilute concentrations of 5-10μM (Morcillo *et al.*, 1999; Mudway and Kelly, 2000).

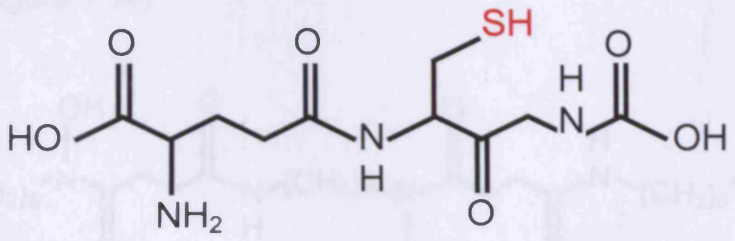

Figure 1.12: *Glutathione structure in its reduced form. Oxidation of two GSH molecules at the cysteine–SH residue, results in a reversible disulphide bridge. GSH also quenches radical species by forming a* $GS^{\bullet}$ *radical.*

1.7.2 *METAL CHELATORS*

The concept of metal chelation originated with the work of Alfred Werner (circa 1893), who proposed the existence of co-ordination chemistry, i.e. the ability of metals to form several bonds to one ligand by virtue of the ligand's possession of more than one electron-pair donor atom (Nobel Lectures, 1966). Since then, chelators have been used in myriad ways, ranging from contaminated soil remediation (via metal abstraction), to toxicity attenuation in clinical therapy and ecotoxicological research (Taylor and Williams, 1995; Tejowulan and Hendershot, 1998; Onikura *et al.*, 2008).

Aminopolycarboxylic ligands, such as ethylene diamine tetra acetic acid (EDTA) and diethylene triamine penta acetic acid (DTPA) are known for their ability to form stable, water-soluble complexes with many metal ions including main group (e.g. Mg and Ca) and TM ions (e.g. Ni, Cu, V, Fe, Cd and Zn; Bell, 1977). Desferoxamine mesylate (DES; Figure 1.13) is predominantly known for its ability to complex Fe, and along with EDTA and DTPA, DES has been shown to ameliorate oxidative stress *in vivo* and *in vitro*. Smith and Aust (1997) treated PM with DES, which resulted in complete inhibition of DNA strand break activity. EDTA is widely used in toxicity identification evaluation and Healey *et al.*, (2006) demonstrated its attenuation of PM-induced mutation in the Ames assay. DTPA was shown to redox-inactivate TM in RTLF by Mudway *et al.*, (2004). All three of these ligands have several carbonyl, OH and NH sites available for electron donation, subsequently forming multidentate ligand-metal complexes (Figure 1.14).

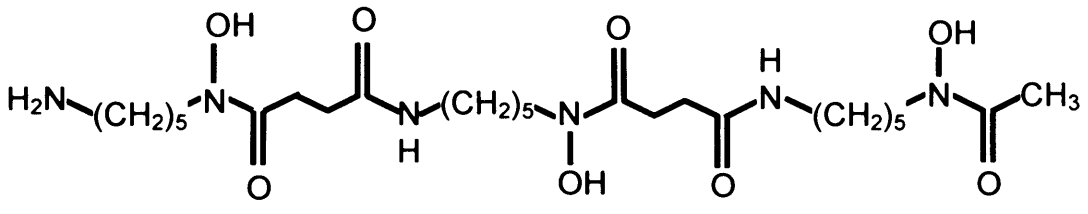

Figure 1.13: *DES has several O electron-donor atoms along its backbone.*

Insoluble resin chelators such as Chelex®-100 (Chelex), α-benzoin oxime (ABO) and dimethylglyoxime (DMG) have also been used in physicochemical

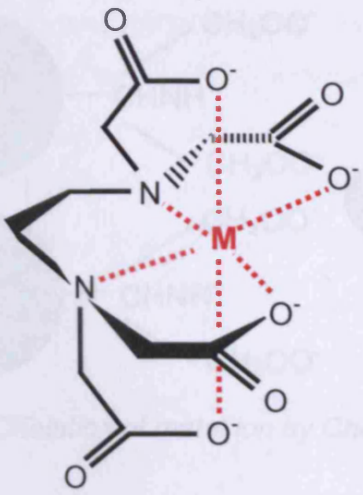

Figure 1.14: *Chelation (hexadentate) of metal (M) ion by EDTA ligand.*

evaluation of environmental effluents (Figure 1.15; Toyota and Nakashima, 1998; Onikura *et al.*, 2008). Chelex is a strong di- and trivalent cation chelating agent of iminodiacetic acid, covalently linked to an inert styrene matrix (Figure 1.16). It has great affinity for a wide variety of metal ions, rendering them biologically-unavailable (Bowles *et al.*, 2006).

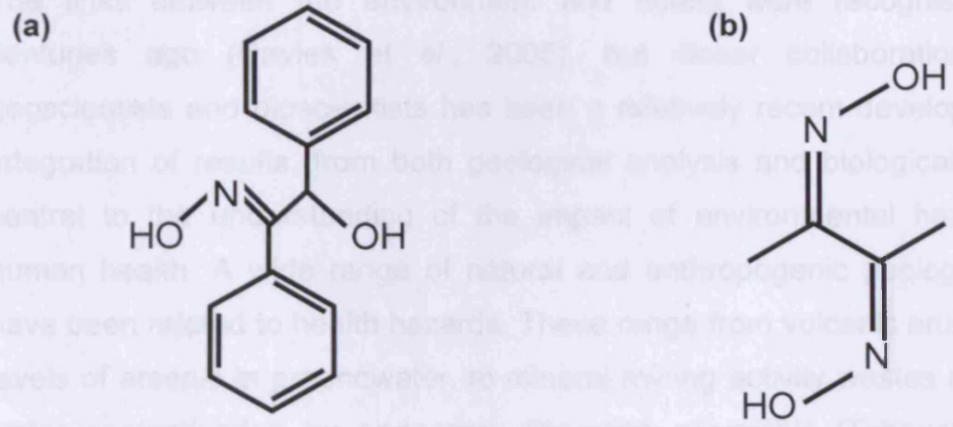

Figure 1.15: *Structures of insoluble chelators* ***(a)*** *ABO and* ***(b)*** *DMG.*

In contrast, ABO and DMG are more specific in metal binding, and have been used to preferentially complex Pb, Cr, Co and Cd (ABO) and Ni (DMG; Toyota and Nakashima, 1998).

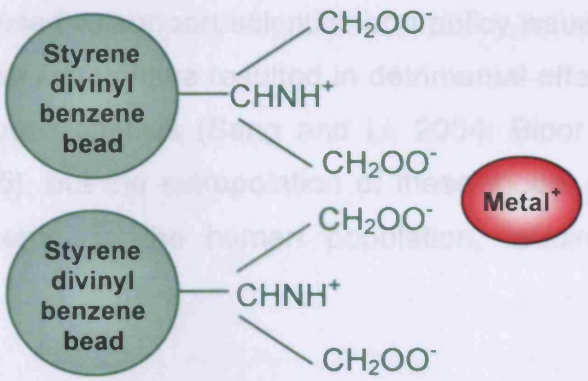

Figure 1.16: *Chelation of metal ion by Chelex at pH 7.4*

The necessary separation of the solid matrix chelate complexes from the sample prior to processing in bioassays, invariably requires greater volumes of sample than the soluble chelator treatments. In contrast, the soluble chelator-metal complex must either remain in the system or undergo further manipulation before the sample can be analysed.

1.8 GEOLOGY AND HEALTH

The links between the environment and health were recognised several centuries ago (Davies *et al.*, 2005), but closer collaboration between geoscientists and bioscientists has been a relatively recent development. The integration of results, from both geological analysis and biological assays, is central to the understanding of the impact of environmental hazards upon human health. A wide range of natural and anthropogenic geological factors have been related to health hazards. These range from volcanic eruptions, high levels of arsenic in groundwater, to mineral mining activity wastes and surface water contamination by endocrine disrupting chemicals (Tchounwou *et al.*, 2003; Fuge, 2005; Slack *et al.*, 2005).

Several geological studies have highlighted the release of pollutants from landfills (Allen, 2001; Slack *et al.*, 2005). Leachate plumes have been detected despite engineered containment practices, whilst other investigations have reported pollution (de la Rosa *et al.*, 2006; Hamoda, 2006). In conjunction with the use of toxicological assays, estimates of the potential bioreactivity of landfill

emissions can be used to support scientific and policy issues. Ecotoxicological studies of landfill leachates have resulted in detrimental effects being observed in murine and aquatic species (Sang and Li, 2004; Bloor and Banks, 2005; Bakare *et al.*, 2005), but the extrapolation of these model systems and dose-response experiments, to the human population, requires interdisciplinary consideration.

Analytical techniques commonly used in geochemistry include Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) to detect metals, and Ion Chromatography (IC) to quantify anions in environmental samples. In addition to these two techniques, Scanning Electron Microscopy (SEM) in conjunction with energy dispersive X-ray microanalysis (EDX) can be used to assess the physicochemical properties of particulate matter (Paoletti *et al.*, 2002; Jones *et al.*, 2006; Godoy *et al.*, 2009), providing information on respirable quality and the chemical composition of these landfill airborne dusts (Chapter 4 and 5). The use of these geochemical analyses of landfill leachates and PM provides information on the chemical constituents of landfill emissions (Øygard *et al.*, 2007; Koshy *et al.*, 2008).

It is also important to find sensitive biological indicators of the potential harm that these emissions may cause following human exposure. Linking toxicological analysis to the geochemical data will help to determine the most accurate degree of toxicity of the environmental emissions produced by landfills. The use of acellular *in vitro* models (e.g. PSA, DCFH assay; see Chapters 2 and 6) to assess bioreactivity of landfill emissions (leachate and PM) can provide an indication of their potential toxicity. This information can in turn, be used in cell-based toxicity assays, and *in vitro* human epidermal and respiratory epithelial models (e.g. ROTAS™ and human tissue equivalents; Chapters 2, 3 and 6). These assays can also be complemented by the use of toxicogenomics to assess alterations in human gene expression, providing an indication of the mechanisms involved in exposure to landfill emissions (Chapter 6).

1.9 LANDFILL SITES OF RESEARCH INTEREST

The waste disposal sites of interest all lie within a 25km radius from Cardiff (Figure 1.17), south Wales, and have been selected due to their different characteristics. Landfill A, a 'containment' landraise site, and landfill B, a 'dilute and disperse' landfill site, are both currently accepting municipal wastes. They have also accepted hazardous waste in the past including car parts, tyres, and clinical wastes (Bathurst, 2002; Paris, 2005; Ling, 2007). Landfill C is a 'containment' landfill site, and although no longer accepting waste, the site has been subject to controversy regarding suspected adverse health effects in the nearby communities (ATSDR, 2003).

Figure 1.17: *Landfill sites of interest in south Wales. Adapted from OS Miniscale™ series. Landfill A = active and partially lined site; Landfill B = active, dilute and disperse site; Landfill C = inactive restored and contained site.*

1.9.1 LANDFILL A

Landfill A (Lamby Way) is located approximately 4km to the southeast of Cardiff City Centre at National Grid reference ST 220 780. The landraise site was

situated on flat lying ground, known as the Wentlooge Levels and was bounded to the north by the Lamby Way road, to the south by the Severn Estuary tidal mudflats, to the west by the mouth of the Rumney river and to the east by the Cors Chrochydd ditch (Figure 1.18). The closest major residential and commercial communities are Splott and Rumney, with an estimated population of approximately 7,000 people living within 1000m of the landraise (George, 2006).

Figure 1.18: *Landfill A, Wales. ☐ Landfill A South restored, unlined phase; ☐ Landfill A East active, lined phase. The River Rumney runs to the west of the site, terminating in the Severn estuary in the south. Open fields and buildings lie to the northeast. Site offices are located in the northern corner of the site, with the weighbridge between the restored and active phases. Adapted with permission of Cardiff City Council (2006). The nearest residential community shown is to the north at Rumney. Adapted from OS Landranger map 171, scale 1:25,000.*

Other significantly close commercial and residential properties include Sea Bank Farm, Mardy House and a scrapper's yard, all within 100 – 250m of the eastern boundary. The drift and solid geology beneath the landraise consists of alluvium clay deposits with no major groundwater aquifers running underneath the site (Cardiff County Council, 1998).

1.9.1.1 LANDFILL A: EAST

After becoming Cardiff's main waste disposal site in 1995, landfill A's capacity was extended eastwards in 1998, whilst adhering to the introduction of legislation requiring the extension to operate as a lined containment site (Bathurst, 2002). The extension covering an area of over 23ha, was constructed as 3 separate cells, which accepted waste sequentially. Cell 1 had reached full capacity by 2002, with Cell 2 scheduled for capping in 2007. Tipping in Cell 3 began in March 2006. The site capacity of the eastern extension was estimated to be over 3 million m^3 MSW. Deposition of refuse occurred in 'raises' that were developed progressively eastwards across the base of the cell.

1.9.1.2 LANDFILL A: SOUTH

Operating from 1978 as a 'dilute and disperse' model, this 38ha section of landfill A was unlined at the base and accepted 2 million m^3 of a wide range of waste types in the past, including municipal, construction and hazardous wastes. The South site waste deposit was built to 25m Above Ordinance Datum (AOD) before being capped and restored to grass cover in 2001. The dilute and disperse nature of this phase meant that until recently, leachate was discharged directly into the Severn Estuary and River Rumney (Cardiff County Council, 1998). Regulatory requirements now mean that leachate is actively pumped from engineered sumps, to a foul-sewer for discharge (Hutchings, J., personal communication, 2005).

1.9.2 LANDFILL B

Landfill B, (Silent Valley) at National Grid Reference SO 185 075, has been in operation since 1981. Access to the site was off the A4046 approximately 20km

to the northeast of Newport, between the communities of Cwm and Waunlwyd (Figure 1.19). Approximately 24,000 people live within 1500m of the landfill (Gray-Jones, 1992). The construction of the landfill was within Cwm Merdogg, a steep-sided valley. The landfill is bounded to the north by the valley, to the south and east by a beech woodland Site of Special Scientific Interest (SSSI), and to the west by a slag heap. The southern edge of the landfill is at approximately 300m AOD, with the estimated waste mass being built to 410m AOD in the northwest.

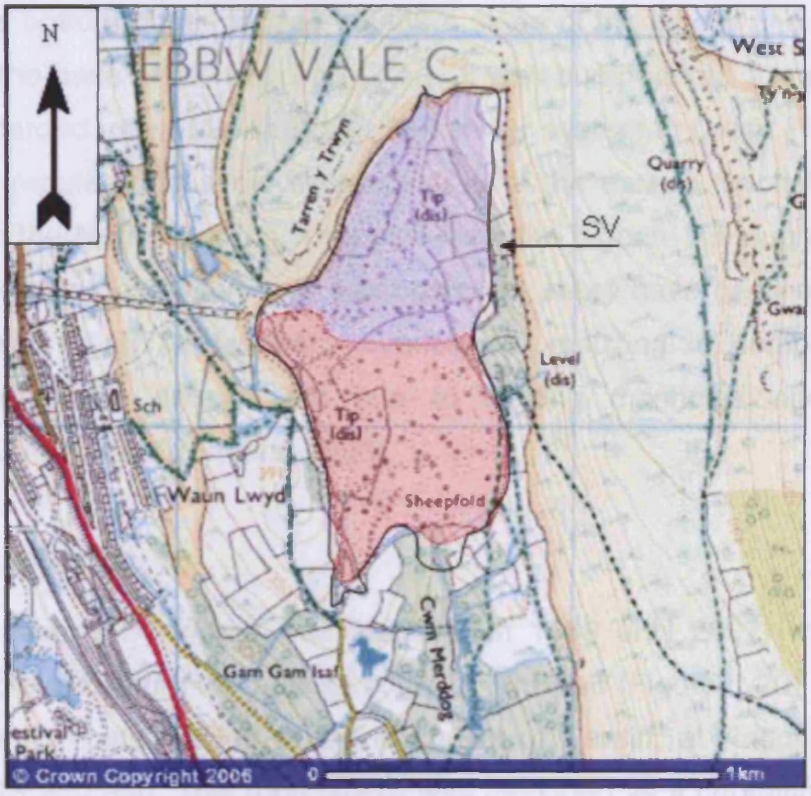

Figure 1.19: *Landfill B, Wales* ■ *Phase 1 waste mass;* ■ *proposed Phase 2. The main residential communities lie to the west at Waunlwyd and to the south at Cwm. Adapted from OS Landranger series 161, scale 1:25,000.*

The site's hydrogeology meant that infiltration and percolation of the groundwater through the landfill was characterised by short transit times, as seen by the flow rates which are monitored out of the southern toe of the landfill (Paris, 2005). Landfill B construction was proposed in two stages. Phase 1, a 'dilute and disperse' area, was active during the study period (2007), covering an area of 20ha and with a capacity of 4 million m^3 waste. Phase 2 was

designated for future development as a containment area, with an estimated 3 million m^3 capacity. Phase 1 lies to the south of the site and was filled in an acellular, uncontrolled manner. However, the current site operators are tipping MSW waste in a cellular process (Paris, 2005).

The site currently accepts municipal waste from the Blaenau Gwent valley communities, but Phase 1 has previously received large quantities of industrial waste from the iron and steel foundries at Ebbw Vale in the 1980s and 1990s (Celtic Technologies, 1995). Leachate was collected from Phase 1 by drainage pipes at the bottom of the capped southern slope of the landfill and flowed into a sump at the base of the site. From here, it was pumped into a lagoon before being discharged into a Welsh Water foul sewer system in Cwm. Flow into the sewer was regulated to avoid overloading, with the excess leachate released directly into the Nant Merddog via a pipe from the lagoon. Although the output into the sewer was monitored by site operators, there have been occurrences of the sewer being inadvertently overloaded, resulting in complaints from private landowners downstream due to leaking manholes and pollution episodes (Ove Arup & Partners, 1996).

1.9.3 LANDFILL C

Landfill C (Nant-y-Gywddon) was active from 1988 until 2002, when it was closed before maximum fill-capacity could be attained (Figure 1.20). The site is situated approximately 25km to the northeast of Cardiff, at National Grid co-ordinates SS 980 940. This rural landfill was designed as a containment facility and was built upon a depression on the steep hillside of Myndd-y-Gelli, an area of high ground lying above the Rhondda Fawr valley in south Wales.

The base of the landfill was estimated to lie 320m AOD, with the waste mass elevated to approximately 350m AOD, covering an area of approximately 10ha. Access to the site was from the northern edge of the site via the B4223, which passed through the town of Gelli. The underlying hydrogeology of the site has been influenced by the legacy of historical mining in the area close to Mynydd-y-Gelli, culminating in a number of disused shafts and adits that may now be

Figure 1.20: *Landfill C, Wales ■ Phase 1 waste mass. The nearest communities are to the north at Gelli and to the south at Clydach Vale. Adapted from OS Landranger series 161, Scale 1:25,000.*

acting as conduits for groundwater, increasing the potential for pollutant dispersal if the engineered liner system fails (Gordon, 2000; Ling, 2007). The head of a local stream, the Nant-y-Gwyddon, originates within the site boundaries, and joins the Afon Rhondda Fawr River at the bottom of the valley. The site began receiving waste in the 1988 but tipping was halted in 2002 before the waste mass reached maximal capacity. Just 1.4 million m^3 waste was believed to have been landfilled, including hazardous as well as municipal waste (Encia, 2004). Similar to landfills A and B, landfill C was planned in two phases, although waste was only ever deposited into Phase 1 before the site was prematurely closed.

The closest residential and commercial communities were Gelli, 500m to the north and Clydach Vale 700m to the south, with a combined population of 20,000 (Fielder, 2000). A significant amount of calcium sulphate in the form of

filter cake was deposited during the mid-1990s, generating large quantities of hydrogen sulphide and resulting in public complaints and concerns over malodour (Purchon, 2001).

1.10 AIMS AND OBJECTIVES

1.10.1 HYPOTHESIS

The hypotheses for this investigation were two-fold:

1. Landfill leachates generate ROS and are cytotoxic to a human skin model.

2. The physicochemical characteristics of landfill PM_{10} differ to urban PM_{10}, thereby affecting the bioreactivity

1.10.2 AIMS AND OBJECTIVES

Landfills are a significant concern in Wales, where the majority of all MSW is disposed via this method. There have been well-publicised cases of possible adverse health effects, mainly birth defects; however, epidemiological studies have largely proved inconclusive (Defra, 2004; Giusti, 2009). There is a current lack of methodical toxicology investigations into the potential risk posed by landfills to the general human population. Therefore, uncertainties regarding the putative effect of landfills upon human health remain. Elucidation of the underlying mechanisms of landfill emissions toxicity could provide a theoretical basis for human health end-points for future risk assessments of landfills.

Exposure routes from landfills are very site-specific, depending on operating methods and hydrogeology. Previous studies have proven inhaled particles of PM_{10} fraction (and smaller) have a detrimental effect on human health, but very little work has conducted on landfill airborne PM. The PM_{10} toxicity has been linked to the generation of ROS and water-soluble contaminants (Greenwell *et al.*, 2002a; Dick *et al.*, 2003; Moreno *et al.*, 2004a).

Leachates contain a vast array of compounds including some trace chemicals that have been designated as carcinogens. Although conditions within landfills tend to be very reducing, preliminary studies have shown neat leachate to be very damaging to plasmid DNA, which is known to be susceptible to free radical ROS attack (Donaldson *et al.*, 1997; Koshy *et al.*, 2007).

The following course of research was undertaken to validate the hypotheses (Figure 1.21):

1. Leachate was collected from three separate landfills in Wales, along with physicochemical data (from landfill site management)

2. Bioreactivity of leachate samples was assessed using the free radical and ROS-sensitive *in vitro* models: Plasmid DNA Scission Assay, oxidation of 2',7'-dichlorodihydrofluorescin, and the effects of metal chelation on this bioreactivity was investigated

3. Microbial toxicity of landfill leachate was investigated with the Rapid On-site Toxicity Audit System

4. PM was collected from landfill and urban sites in 2007 and 2008, and Scanning Electron Microscopy utilised to assess properties of these PM samples

5. Physicochemical properties of landfill and urban PM samples were characterised by the use of Inductively Coupled Plasma-Mass Spectroscopy to measure metal content and Ion Chromatography to measure anion content

6. Bioreactivity of PM samples was assessed using the ROS-sensitive *in vitro* model: Plasmid DNA Scission Assay, and the effects of metal chelation on this bioreactivity was investigated

7. Following identification of select toxic samples, the dermal and respiratory epithelia from human tissue equivalents were used to investigate the *in vitro* cytotoxicity (leachate and PM) and toxicogenomics of landfill PM versus urban PM.

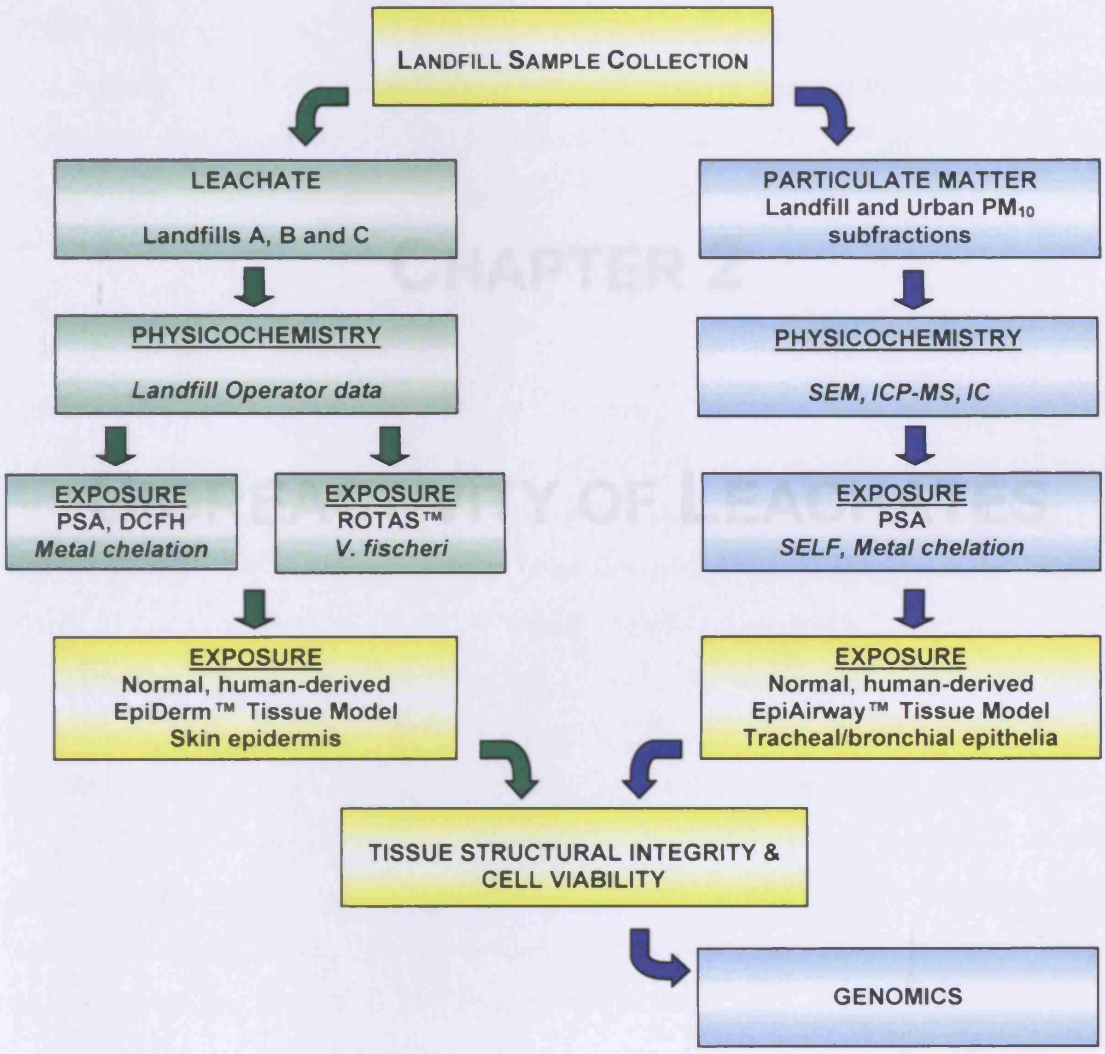

Figure 1.21: *Flowchart of the project aims involved in evaluation of toxic potential of landfill leachate and PM emissions, in acellular and human tissue equivalent models. Landfills were sampled for leachate and PM_{10}, for evaluation by ROS-sensitive assays. Physicochemistry data was gathered from landfill operators (leachate) or generated in-house by standard chemical methods. Nascent leachate was evaluated for cytotoxicity with the EpiDerm™model; landfill PM_{10} toxicogenomics was compared to the effect of urban PM_{10} in the EpiAirway™model.*

CHAPTER 2

BIOREACTIVITY OF LEACHATES

2.1 INTRODUCTION

The most common metals present in MSW leachate include Fe and Mn (present in the mg/L level; Alkalay *et al.*, 1998), both of which are well known for their redox properties. Copper, Pb, Zn, Ni, Cr and Cd are also major leachate metals. Since landfill leachate tends to be highly reducing (Christensen *et al.*, 2001), TM metals are present in redox states conducive to participation in Fenton chemistry. Previous studies have revealed that landfill leachates can have toxic effects upon various ecological levels, including plants, microbial organisms, algae, and invertebrates such as *Daphnia magna* and *Artemia salina* (Schrab *et al.*, 1993; Bernard *et al.*, 1997; Baun *et al.*, 2004; Sang *et al.*, 2004).

Increased fish lethality and abnormal embryo hatching as a consequence of untreated leachate exposure, also supports the high aquatic toxicity of MSW leachate (Osaki *et al.*, 2006). The role of free radicals in the toxic and genotoxic potential of landfill leachates in all these systems is an emerging hypothesis in this field (Bortolotto *et al.*, 2009). Elevated oxidative damage (measured by lipid peroxidation and depleted antioxidant status), has been detected in the brains and livers of mice orally administered MSW leachate (Li *et al.*, 2005) and genotoxicity, in the form of chromosomal aberrations, has been reported (Li *et al.*, 2004). Leachate-induced genotoxicity in murine bone marrow was also detected by Tewari *et al.*, (2006), using the Comet assay for DNA-strand breaks. In both investigations, oxidative stress mediated by free radical damage, was postulated to be the mechanism of action.

In the UK waste management industry, licenses for discharge consent of landfill effluents are based on the physicochemical parameters of leachate. The biological assessment of leachate is not required, and as such, there is a lack of knowledge regarding its toxicity. The assessment of the *in vitro* bioreactivity of various landfill leachates will contribute to the current field of research. Accordingly, bioreactivity was gauged in terms of the oxidative capacity of the samples and was evaluated using a number of assays (Koshy *et al.*, 2007) that were designed to assess reactive oxidant species (ROS).

The ultimate objective of the work described in this chapter was to determine if landfill leachates that were proven to be bioreactive and damaging in the PSA and ROTAS™ assay, would cause cytotoxic effects in human skin, thereby posing a potential hazard in occupational dermal exposure. There is little abstraction of groundwater for domestic consumption in the UK. Therefore, skin exposure can be considered a possible result of leachate generation, where dermal exposure to leachate vapour or aerosol during spray irrigation of leachate and exudations of leachate directly from the waste mass, are possible hazards. Although regulations in the UK aim for containment principle, requiring adherence to modern landfill engineering standards, leachate leaks may yet pose a risk to bystanders (Figure 2.1).

Figure 2.1: *Example of leachate migration away from lined, active study site, landfill A, towards an access road. Note the lack of vegetation (brown areas) along the leak.*

The aims of this chapter were to investigate leachate with respect to its possible mechanisms of toxicity; this was achieved by:

- Collecting leachates from the three study landfills outlined in Chapter 1

- Screening MSW leachates from different landfills with the ROS-sensitive plasmid DNA scission (PSA) and 2',7'-Dichlorodihydrofluorescin (DCFH) assays
- Evaluating the role of leachate metals in the samples established as bioreactive in the PSA and DCFH assays, by incorporation of metal chelators into the systems
- Investigating the cytotoxic effects of the most bioreactive leachates on EpiDerm™-200, a commercial 3-D human skin model.

2.2 METHODS

2.2.1 MATERIALS AND SOURCES

All sources are UK-based unless otherwise stated.

Material:	Source:
α-Benzoin Oxime	Sigma-Aldrich
10ml Syringes	Terumo, Leuven, Belgium
18 MΩ Water	In-house, Cardiff University
Agarose Tablets 0.5g	Bioline
Cary Eclipse Fluorimeter	Varian Instruments, CA, USA
Cary Eclipse Software	Varian Instruments, CA, USA
Chelex-100 (Sodium)	Sigma-Aldrich
Cuvettes (10 x 10 x 45mm)	Greiner BioOne
2',7'-Dichlorodihydrofluorescin Diacetate	Sigma-Aldrich
Dionex DX-80	Dionex
Distilled Water	In-house, Cardiff University
Dimethyl Glyoxime	Sigma-Aldrich
Electrophoresis Maxi Tank	Helena Biosciences
Endohm Chamber ENDOHM-12	World Precision Instruments
EpiDerm™-200	MatTek Corporation, USA
Eppendorfs 1.5ml	Anachem
Eppendorf Incubation Block	Grant Instruments
Ethidium Bromide 10mg/ml	Sigma-Aldrich
Genetools Software	Syngene
Hydrogen Peroxide 30% (w/w)	Sigma-Aldrich

Iron (II) Sulphate	BDH AnalaR
Methanol (HPLC grade)	Fisher Scientific,
Molecular Biology Reagent Water	Sigma-Aldrich
MTT	Sigma-Aldrich
MTT Assay Solubilisation Solution	Sigma-Aldrich
Opsys Plate Reader (96 well)	Dynex Technologies
Orange/ blue Loading Dye 6x	Promega
ΦX174 RF DNA	Promega
Phosphate Buffered Saline (Ca/Mg)	Sigma- Aldrich
Probes (Field Measurement)	HANNA Instruments
Pst I Restriction Enzyme Kit	Promega
PVDF 0.45µm Filter Discs	Millipore, Cork, Ireland
PCR tube DNAase/RNAase- free, 200µl	Alpha labs
Sodium Hydroxide	Fisher Scientific
Sodium Dihydrogen Orthophosphate	Fisher Scientific
Thermo X7 ICP-MS	Thermo Electron Corporation
Tris-Borate-EDTA (10x).	Sigma-Aldrich
Tris-HCl, 1M, pH 8.0	Sigma-Aldrich
UVP Biospectrum Imaging System	Ultraviolet Products
Vortex Genie 2	Jencons
Waterra Single Valve Bailer	Waterra
CA 0.22µm Filter Discs	GE Healthcare Life Sciences

2.2.2 SAMPLING METHODOLOGY

Samples (Table 2.1) were collected in November 2005 and at regular intervals during 2006 – 2007. Sample collection techniques varied between sites (Figure 2.2), but all leachate samples were collected in 250ml High Density Polyethylene (HDPE) bottles with minimal headspace, filtered at 0.45µm within 2h of collection, and stored at 4°C in the dark until use.

PARAMETER	LANDFILL A	LANDFILL B	LANDFILL C
Type	Active; partially contained	Active, dilute & disperse	Restored; contained
Operation since	1978-current	1981-current	1988-2002
Waste type (in addition to MSW)	Mixed Industrial, Chemical, Bulky	Iron & Steel Industrial	Industrial, Filter cake
Collective Sump	A1	B1	C1
Restored Phase	A2	B2	C2
Active Phase	A3	n/a	n/a
Leachate Reservoir	n/a	B3	C3
Leachate volume discharged (m^3/year)	200,000*	250,000*	36,250

Table 2.1: *Sampling well characteristics of landfill study sites.* ***Landfill A:*** *Mixed Industrial waste can be from a variety of sources, ranging from combustion wastes, e.g. clinker from power stations, discarded equipment and mineral wastes. Chemical waste can be derived from the manufacture of wide range of man-made products, e.g. rubber and plastics, metal fibres, oils and solvents. Bulky wastes include discarded equipment, car parts, construction and demolition wastes.* ***Landfill B:*** *Iron & steel industrial waste is primarily slag, a by-product of iron ore reduction to steel.* ***Landfill C:*** *Filter cake in the form of hydrated calcium sulphate is also known as gypsum.* **Estimated values based on landfill operator data during 2006. n/a Indicates samples were not collected (well absent or inaccessible).*

Figure 2.2: *Leachate collection techniques applied during the study.* ***(a)*** *Samples of leachate in HDPE bottles;* ***(b)*** *Two-litre polypropylene vessel used to collect leachate from sampling wells at landfill A;* ***(c)*** *Sampling from landfill B pipe B1;* ***(d)*** *Dip-sampling at B3 below the fixed conductivity meter;* ***(e)*** *Teflon Waterra™ bailer used to collect leachate from landfill C restored phase C2;* ***(f)*** *One-litre cubic steel container used to collect leachate from collective sump C1.*

2.2.2.1 LANDFILL A

Landfill A samples were collected from January 2006 at 30 – 45 day intervals by lowering a two-litre polypropylene vessel into each well. Samples were taken from the active, east phase at a central collection sump (A1; Figure 2.2.b), and samples of the restored, south (capped) phase leachate were taken from well A2.

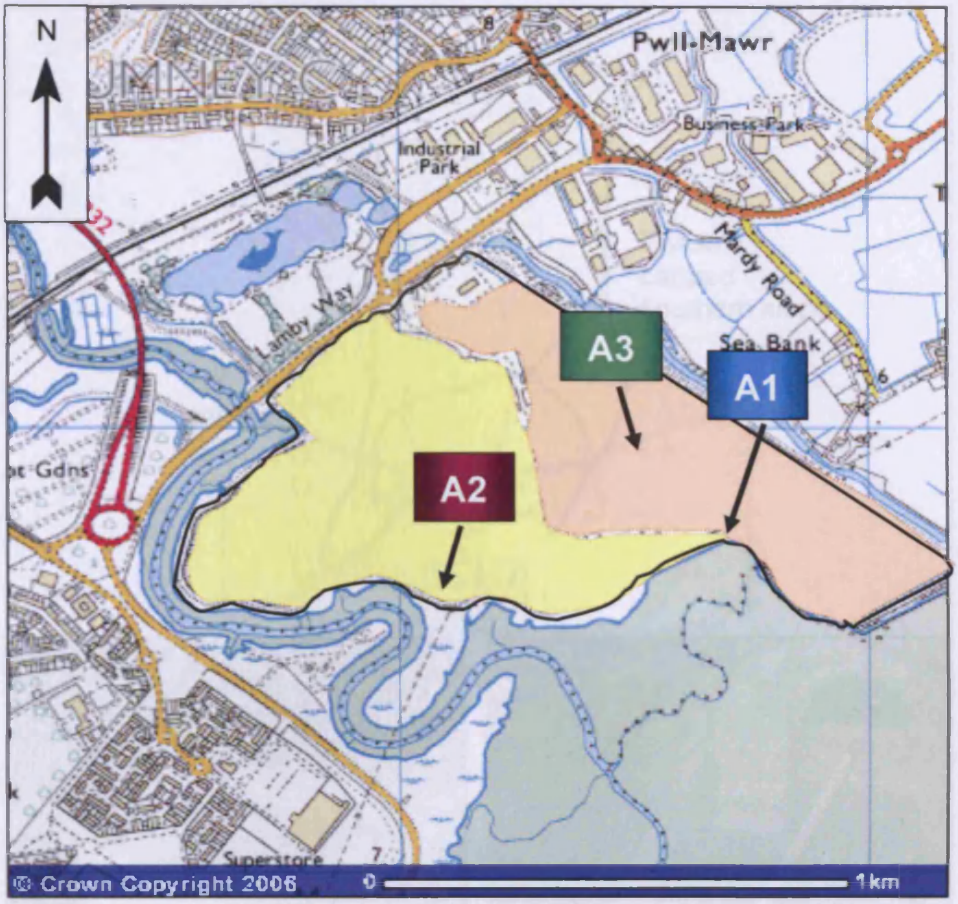

Figure 2.3: *Landfill A sampling points from active phase and capped, restored phase. collective sump A1, active phase A3; capped, restored phase A2.*

2.2.2.2 LANDFILL B

Landfill B leachate samples were collected in November 2005, and from January 2006 directly into HPDE sample vessels from the inflow of pipe B1 into the sump and from pipe B2 inflow into the leachate reservoir (B3; Figures 2.2.c and 2.4). Reservoir B3 was divided into upper and lower sections. Leachate was dip-sampled from the upper tank of B3, using the location of a permanently mounted conductivity meter at the south-western edge of B3 as a marker for consistency (Figures 2.2.d and 2.4).

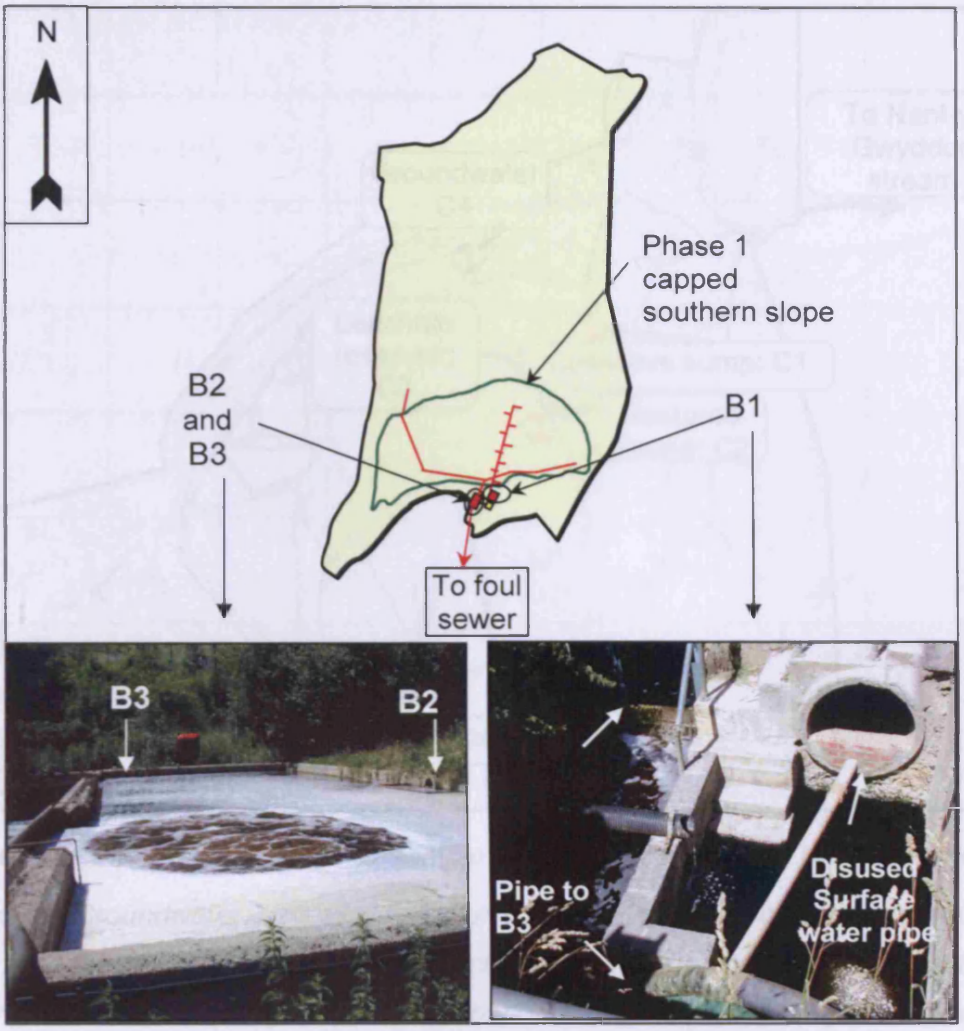

Figure 2.4: *Landfill B sampling points B1, B2 and B3 indicated in the south of the site.* — *Leachate pipelines through the drainage layer of the southern slope fed into separate B1 and B2 pipelines;* — *Clay cap on the restored southern slope was designed to prevent water ingress;* — *Clay boundary of the landfill site. Effluent was discharged from the site from the bottom of B3 into a foul sewer system (not illustrated). Refer to Figure 1.19 for scale.*

2.2.2.3 LANDFILL C

Landfill C groundwater was sampled from C4 to the north of the waste mass. Leachates were collected from the leachate lagoon (C3) and from sump C1 using on-site equipment kindly provided by the site operators, Amgen Cymru Ltd. The equipment consisted of a one litre steel thick-walled cubic container supplied by the site management (Figure 2.2.e). Leachate samples were collected from a gas well (C2) towards the east of the restored waste mass. Teflon bailers were used to sample from C2 and C4 (Figures 2.2.f and 2.5).

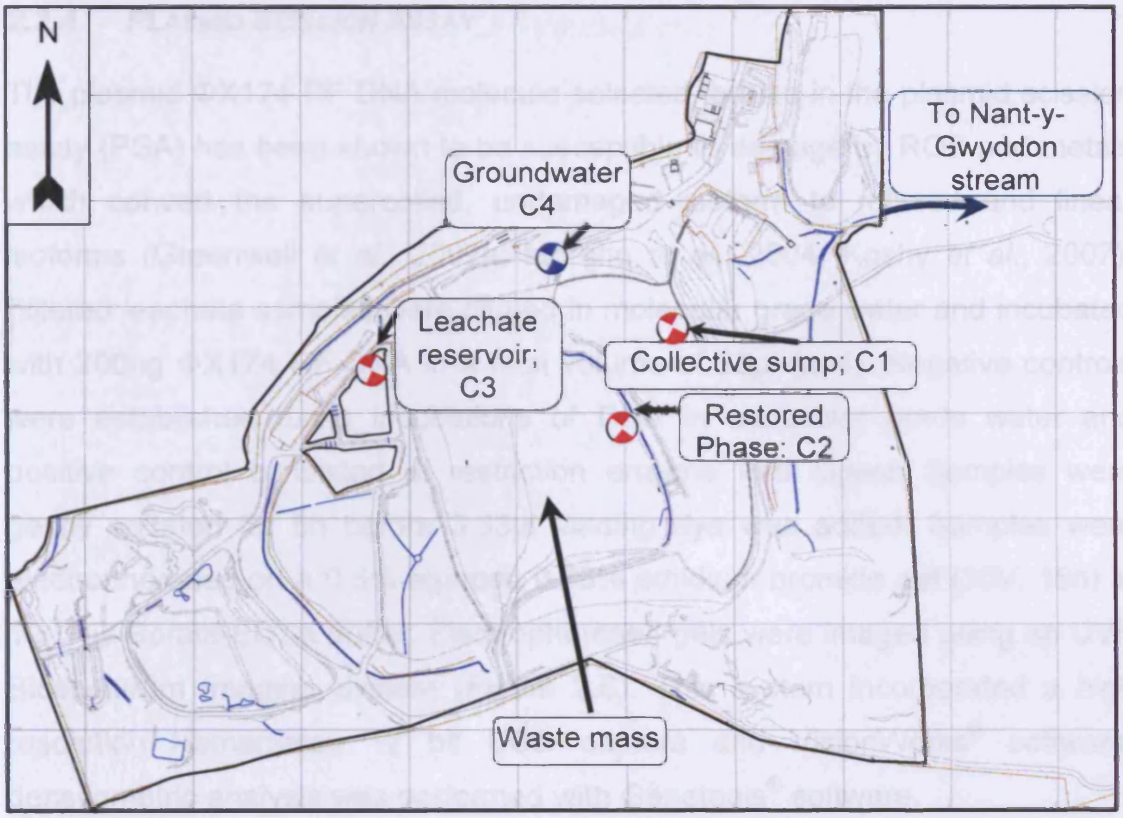

Figure 2.5: *Site plan of landfill C sample collection points.* *Leachate collection points;* *Groundwater borehole;* *Surface water drainage channels carried water away from the site to the head of the Nant-y-Gwyddon stream to the northeast of the site;* *Leachate drainage channels transported leachate to the lagoons in the west of the site. Refer to Figure 1.20 for scale.*

2.2.3 PHYSICOCHEMICAL ANALYSIS

All data were provided by the landfill operators after sample analysis by contracted and certified laboratories (Barnett, A., Chard, N., Ling, S., personal communications, 2007). The parameters assessed in this study included ammoniacal-nitrogen (NH_4^+-N), pH, COD, BOD, suspended solids (SS), Cl, Mecoprop (persistent pesticide residue; MCPP), m&p-xylene (organic solvent) and metals (Cd, Ni, Cr, Zn, Cu, Fe and Pb). Ion chromatography was performed at Cardiff University to analyse samples for Cl^- where data were not available.

2.2.4 *Plasmid Scission Assay*

The plasmid ΦX174 RF DNA molecule selected for use in the plasmid scission assay (PSA) has been shown to be susceptible to damage by ROS and metals which convert the supercoiled, undamaged isoform to relaxed and linear isoforms (Greenwell *et al.*, 2002a; Moreno *et al.*, 2004; Koshy *et al.*, 2007). Filtered leachate samples were diluted in molecular grade water and incubated with 200ng ΦX174 RF DNA in a final volume of 20µl (n=4). Negative controls were established using incubations of DNA in molecular grade water and positive control consisted of restriction enzyme PstI digest. Samples were gently agitated for 6h before 3.33µl loading dye was added. Samples were electrophoresed on a 0.6% agarose 0.25% ethidium bromide gel (30V, 16h) in 1 x Tris-Borate-EDTA buffer. Electrophoresed gels were imaged using an UVP Biospectrum Imaging System (Figure 2.6). This system incorporated a high resolution Hamamatsu 12 bit CCD camera and VisionWorks® software; densitometric analysis was performed with Genetools® software.

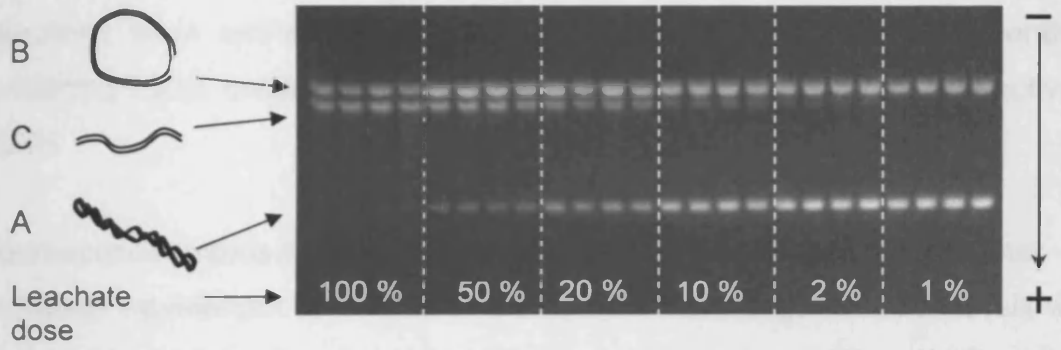

Figure 2.6: *Plasmid scission assay of ΦX174 RF DNA incubations with various concentrations of landfill leachate, with direction of DNA migration from cathode to anode indicated. Conformations at varying levels of progressive damage; A: intact undamaged supercoil, B: relaxed open coil, containing single-strand breaks, C: severely damaged linearized DNA (Koshy et al., 2007).*

The relative amount of damaged DNA (relaxed open circle form plus linearized form) in each lane was calculated as a percentage of the supercoiled intact DNA. Gels with less than 10% damage in the water controls were accepted for analysis.

2.2.5 2',7'-DICHLORODIHYDROFLUORESCIN ASSAY

The 2',7'-Dichlorodihydrofluorescin (DCFH) *in vitro* assay used to determine the oxidative capacity of different leachate samples. It exploits the oxidation of DCFH to 2',7'-dichlorofluorescin (DCF); a strongly fluorescent moiety (Figure 2.7). The compound was sourced as 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA, Sigma-Aldrich, UK), due to the increased stability of the precursor, which was chemically cleaved to the working form of DCFH (Figure 2.7). This compound has been widely used as a marker of cellular oxidative stress and as an indicator of reactive species formation (Hung and Wang, 2001; Gomes *et al.*, 2005; Koshy *et al.*, 2007).

A working solution of DCFH was prepared from DCFH-DA using the method of Cathcart *et al.*, (1983). In brief, 2ml 0.01M NaOH was added to 0.5ml 1mM DCFH-DA in methanol, and this hydrolysate was allowed to react at ambient temperature in the dark for 30min prior to neutralisation with 10ml 25mM NaH_2PO_4 (pH 7.4), providing a 40µM stock solution of activated DCFH. Reactions were carried out at 37°C for 25min in light-resistant eppendorfs, containing 750µl undiluted leachate and a final concentration of 1µM activated DCFH.

Fluorescence intensity was measured in a Cary Eclipse Fluorimeter with excitation wavelength at 485nm and emission wavelength at 530nm (slit width 10nm). Measuring the fluorescence of each pre-incubation confirmed the absence of interfering organic compounds in leachate samples. All samples emitted background levels of fluorescence prior to addition of DCFH. Parallel incubations were performed with 1.485ml 25mM NaH_2PO_4 (pH 7.4), containing a final concentration of 1µM activated DCFH. This provided a measure of the extent of dye auto-fluorescence (LeBel *et al.*, 1992). In order to correlate the fluorescence of samples to "equivalents of H_2O_2" (Hung and Wang, 2001), an indicator of oxidative stress, a linear calibration curve of H_2O_2 was generated. All calibration reactions were performed in 40mM Tris-HCl pH 7.4 containing 1µM activated DCFH solution. This mixture was spiked with 0.1 – 300µM H_2O_2 before initiation of the reaction by addition of 10µM $FeSO_4$ in a final volume of

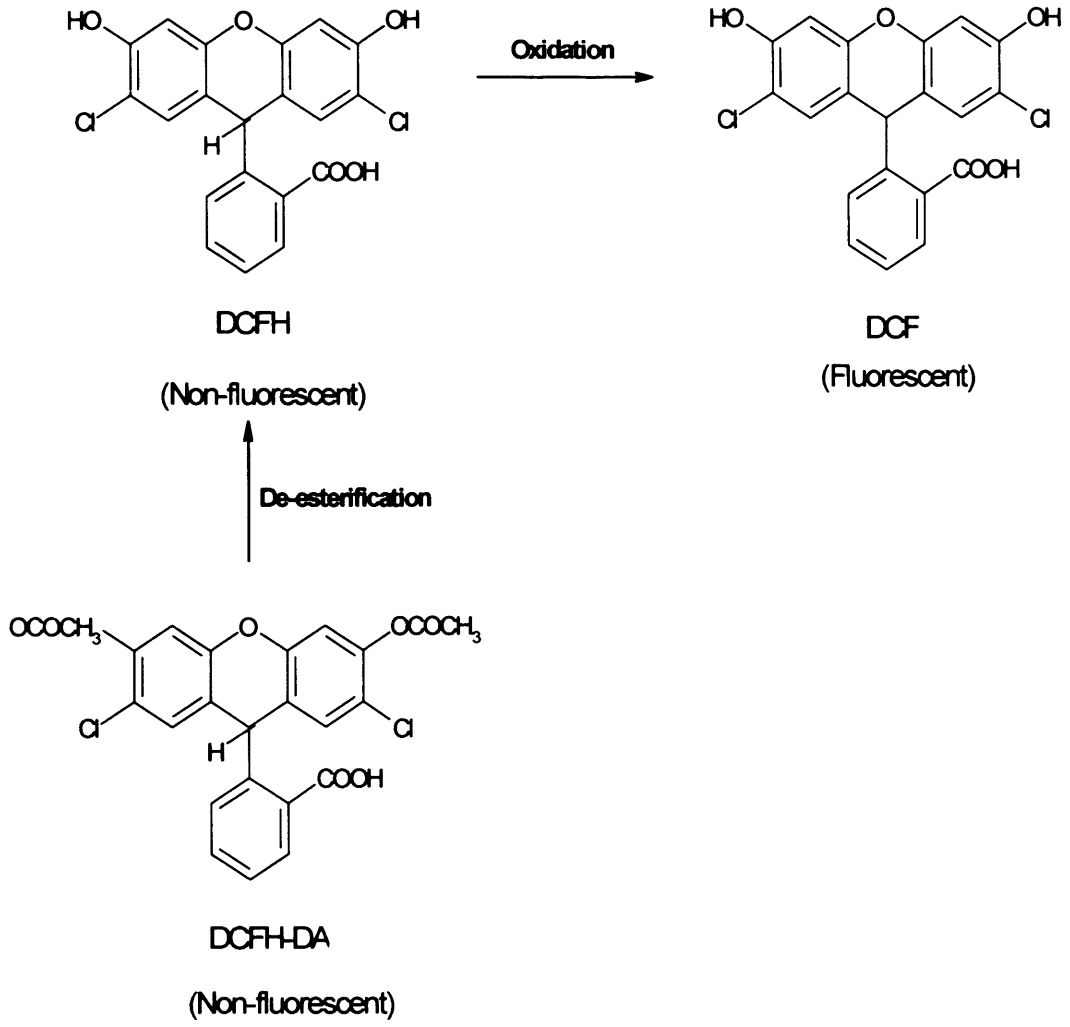

Figure 2.7: *Chemical activation of 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) to 2',7'-dichlorodihydrofluorescin (DCFH) and the in vitro formation of the fluorogenic 2',7'-dichlorofluorescin (DCF) moiety.*

1.5ml. The Fe^{2+} reacts with H_2O_2 (Fenton reaction), leading to hydroxyl radical formation ($^{\bullet}OH$), which accelerates the oxidation of DCFH (Myhre *et al.*, 2003).

2.2.5 Preparation of Metal Chelators for PSA and DCFH Assays

The sodium form of Chelex-100 (Chelex), α-benzoin oxime (ABO), and dimethylglyoxime (DMG) were used in batch chelation of bioreactive leachate samples at a concentration which had been demonstrated to be 100% damaging in the PSA. All treatments were carried out for 6 hours at 4°C; reagents were analytical or molecular grade. The leachate was gently agitated with the sodium form of Chelex resin, ABO or DMG (50mg/L). Following

incubation, the chelator mass was pelleted by centrifugation (12000 x *g*; 2min) and the leachate supernatant was decanted from the resin for use in the PSA and DCFH assays. For the soluble chelator treatments (DTPA, EDTA), leachates were spiked with a neutralised stock chelator solution, prepared in molecular grade water, to obtain a final DTPA or EDTA concentration of 10mM in leachate. The soluble chelators remained in the leachate for the duration of the evaluation.

2.2.5.1 Leachate Physicochemical Analysis

In this case, a MSW leachate from the active landfill A (collective sump A1 collected in March 2008) was selected as a suitable effluent for evaluation of metal-induced bioreactivity. Probe field measurements included temperature, pH and oxidation/reduction potential (HI991003 meter), electrical conductivity and Total Dissolved Solids (TDS) were measured on samples diluted with 18MΩ water at a ratio of 1:5 (HI98311 meter). All probes were calibrated according to manufacturer's instructions prior to use. Ion chromatography for Cl^- and SO_4^{2-} was performed at Cardiff University (Dionex, Model DX-80), whilst data for ammoniacal-nitrogen (NH_4^+-N) was provided by the landfill site operator following analysis by a contracted and UKAS-certified laboratory. Samples for metal analysis were preserved with 1N HNO_3, and analysed for major and trace elements by ICP-MS. A Thermo Elemental X7 High Resolution ICP-MS was used to quantify Na, Mg, K, Al, Ca, Fe, V, Cr, Mn, Ni, Cu, Zn, As and Pb.

2.2.6 EpiDerm™ Exposure

2.2.6.1 EpiDerm™ Tissue Culture

The skin model was purchased from MatTek Corporation (USA) as a fully-differentiated tissue, approximately 10mm in diameter and 1mm thick. The cells were derived from normal, human, epidermal keratinocytes of neonate foreskin. These were cultured over a period of 2-3 weeks as individual tissue culture inserts, in order to develop the multi-layered structure (Figure 2.8). The final (ready-to-use) tissue inserts were transported in a 24-well plate at 4°C and upon receipt, removed from the shipment gel and placed in 0.9ml media in

6-well plates in 37°C, 5% CO_2 for 24h acclimatisation prior to dosing.

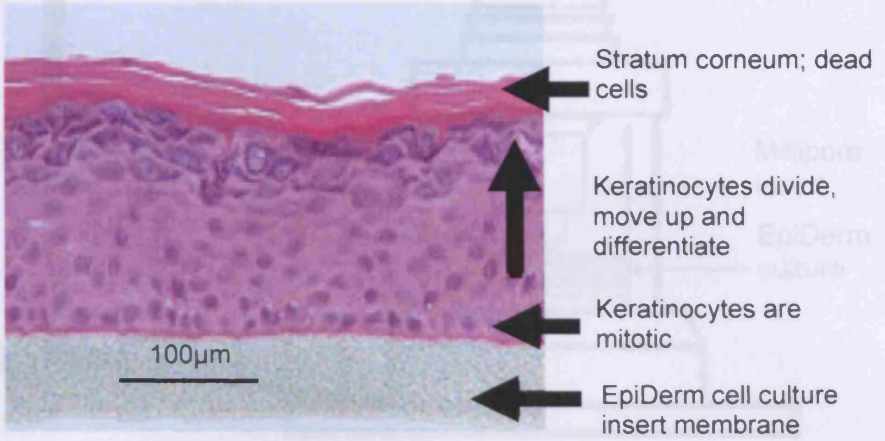

Figure 2.8: *Cross-section of an EpiDerm™ tissue cross-section. Adapted from Aardema et al., (2005).*

2.2.6.2 *Leachate Preparation and Exposure*

Samples from the active phase of all three landfills in January and February 2007 were collected as described in Section 2.2.2, before being filtered through 0.22µm filter discs. After the acclimatisation period of the EpiDerm™ tissue was complete, 100µl of warm (37°C), undiluted leachate or PBS (negative control) was applied to the apical surface of the EpiDerm™ and inserts were returned to the incubator for an exposure period of 24h (n=6). Upon completion of the exposure period, the leachate was aspirated and the apical surface rinsed with 350µl warm PBS.

2.2.6.3 *Tissue Integrity*

Tissue integrity was determined by the trans-epithelial electrical resistance (TEER) prior-to, and following dosing. The TEER values of each tissue insert were evaluated using the Endohm™ chamber (Figure 2.9). The chamber was first filled with 1ml of PBS solution to cover the anode. The tissue insert was placed inside the Endohm chamber and 350µl of PBS solution was placed onto the apical surface of the EpiDerm™. The cathode was placed on top of the insert and the resistance through the insert obtained (ohms; Ω). Background resistance values were obtained with a tissue-free Millipore insert containing PBS, at the beginning and every 6 readings.

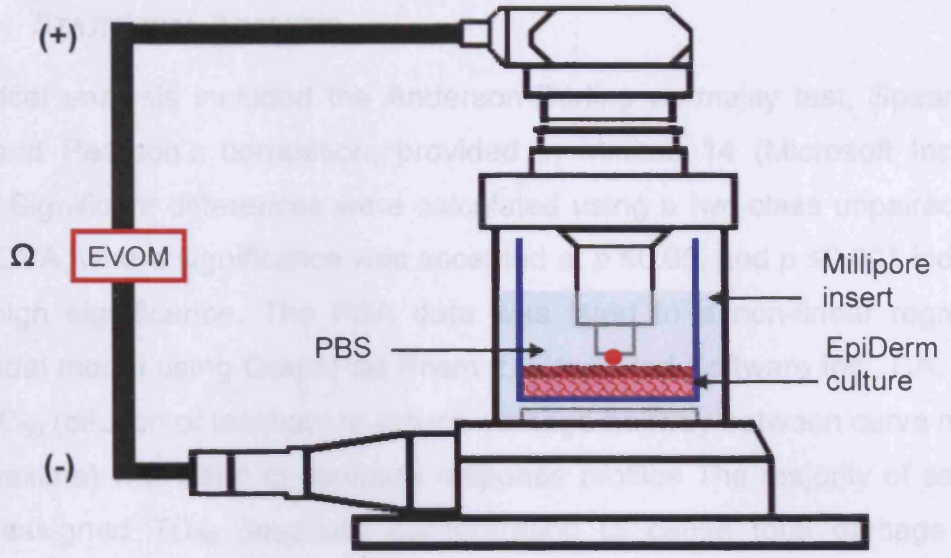

Figure 2.9: *TEER measurement with an Endohm™ chamber linked to EVOM meter. The cell insert was placed inside the Endohm-12 chamber (as indicated).*

2.2.6.4 CELL VIABILITY

Selected inserts (n=3) of each leachate dose and PBS exposure, were processed by the MTT viability assay. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to measure mitochondrial activity as an indication of cell viability. Post-dose rinsed inserts (Section 2.2.6.2) were placed in 300µl fresh media containing 1mg/ml MTT, protected from light, and returned to 37°C, 5% CO_2 for a 3h loading period. During this incubation, soluble MTT (yellow) crossed the cell membrane and entered the mitochondria, where the MTT was reduced to insoluble purple crystals by a dehydrogenase involved in the electron transport chain in live cells. After the dye loading period, the inserts were transferred to a 24-well plate and 2ml MTT solubilisation solution was added. This solution comprised an acidic detergent which lysed the cells and dissolved the MTT crystals to a purple solution. This plate was wrapped in foil and laboratory film as protection from evaporation and light. The extraction was allowed to proceed for 24h (RTP) after which, the inserts were removed; the contents of each well were thoroughly mixed and then diluted 1:10 (v/v) in solubilisation solution before the absorbance was read at 540nm in an Opsys™ 96-well plate reader.

2.2.6 *STATISTICAL ANALYSIS*

Statistical analysis included the Anderson-Darling normality test, Spearman's rank and Pearson's correlation, provided in Minitab 14 (Microsoft Inc, WA, USA). Significant differences were calculated using a two-class unpaired t-test or ANOVA; where significance was accepted at $p \leq 0.05$, and $p \leq 0.001$ indicated very high significance. The PSA data was fitted to a non-linear regression sigmoidal model using GraphPad Prism 2 (GraphPad Software Inc., CA, USA). The EC_{50} (dilution of leachate to induce damage halfway between curve minima and maxima) was used to compare response profiles The majority of samples were assigned TD_{50} (leachate concentration to cause total damage 50%) values, but where sample maximum bioreactivity fell below 50%, a TD_{25} (total damage 25%) value was generated as listed in Table 2.2.

2.3 *RESULTS*

2.3.1 *PHYSICOCHEMICAL ANALYSIS*

The results for basic physicochemical analysis of the leachate samples are presented in Tables 2.2 and 2.3. Data for NH_4^+-N, pH, COD, BOD, SS, Cl^-, MCPP and m&p-xylene were acquired for the majority of leachates assessed by PSA. Heavy and transition metals analysed included Cd, Ni, Cr, Zn, Cu, Fe and Pb. The relationship between these parameters and bioreactivity, as measured by the PSA, was investigated with Spearman's Rank Correlation Analysis and a significant negative association ($p < 0.05$) was observed between TD_{50} and MCPP, with a correlation coefficient of -0.817.

Landfill Leachate	Month	PSA		Equivalent H_2O_2 µM	NH_4^+- N mg/L (0.5)[a]	pH	COD mg/L O_2	BOD mg/L O_2	SS mg/L	Cl^- mg/L (250)[a]	MCPP* µg/L (10)[a]	m&p xylene µg/L (500)[a]
		TD_{25}	TD_{50}									
C1	March	-	1	182	1230	8.1	2640	34	68	1120	-	-
A1	September	-	2	290	-	6.9	-	-	-	945[b]	26.90	11.90
A3	February	-	4	45	606	-	-	-	-	812[b]	-	-
B3	February	-	6	291	1.21	8.6	-	19	58	586	-	-
A1	February	-	7	85	589	-	-	-	-	922	30.30	25.50
C2	January	-	8	154	-	-	-	-	-	1102[b]	-	-
A1	April	-	8.5	66	704	-	-	-	-	1270	25.30	24.80
A2	April	-	9	95	1	-	-	7	-	-	-	-
B3	May	-	10	325	88.8	8.3	-	9	12	229	-	-
A1	June	-	16	260	658	7.0	-	-	-	876	25.70	20.40
B3	March	-	17	124	1.68	8.4	-	19	6	474	-	-
A2	February	-	18	52	1.1	-	-	1	-	146[b]	-	-
C1	September	-	18	86	1240	7.6	2520	196	72	1340	-	-
C2	September	-	18	156	-	7.0	-	-	-	1206[b]	-	-
A1	October	-	20	84	367	6.8	-	-	-	487	-	6.31
A3	October	-	21	67	427	7.2	-	-	-	1023[b]	-	-

Table 2.2: *Bioreactivity, physical and chemical characteristics of the leachate samples listed in order of decreasing PSA toxicity. Data kindly provided by landfill operators. TD_{50} = leachate concentration at which 50% plasmid DNA damage occurs. [a]Values in parentheses represent WHO drinking water quality guidelines (WHO, 2008). [b]Analysis was performed at Cardiff University. *MCPP (mecoprop) pesticide residue has been used by previous investigators as an indicator of organic pollutants; MCPP and Equivalent H_2O_2 showed significant negative correlations with PSA TD_{50} values (r = -0.82 and -0.72, respectively). Inoperative landfill collective sump (C1) exhibited consistently high NH_4^+- N, COD, Cl^- and BOD. Reservoir leachate B3 exhibited fluctuating NH_4^+- N and all other leachate BODs were generally low.*

Landfill Leachate	Month	PSA		Equivalent H_2O_2 µM	NH_4^+- N mg/L (0.5)[a]	pH	COD mg/L O_2	BOD mg/L O_2	SS mg/L	Cl^- mg/L (250)[a]	MCPP µg/L (10)[a]	m&p xylene µg/L (500)[a]
		TD_{25}	TD_{50}									
C1	June	-	22	70	840	8.1	2780	-	67	1300	-	-
A2	June	-	23	69	-	7.5	75	1	148	26[b]	-	-
C1	July	-	23	94	-	7.4	-	-	-	-	-	-
C2	October	-	23	-	-	7.2	-	-	-	1081[b]	-	-
B3	June	-	26	63	1.13	8.5	-	-	31	631	-	-
C3	January	-	26	44	91.6	8.2	291	17	452	193	-	-
B3	July	-	27	72	327	8.5	-	-	26	774	22.20	-
A1	July	-	28	55	597	7.2	-	-	-	840	27.40	23.70
B3	October	-	33	-	156	8.4	-	-	18	451	10.80	-
B3	April	-	35	-	361	8.5	-	-	84	633	21.00	-
C2	July	-	35	72	-	7.2	-	-	-	1465	-	-
C3	July	-	39	-	-	8.5	536	14	266	554	-	-
A1	August	-	41	51	-	7.0	-	-	-	45	18.70	13.70
C1	January	-	45	43	650	8.2	1860	211	-	952	-	0.96
B2	November	-	46	56	140	8.0	-	15	19	413	-	-
B3	August	-	47	86	359	8.5	-	15	26	780	-	-

Table 2.2 continued: *Bioreactivity, physical and chemical characteristics of the leachate samples listed in order of decreasing PSA toxicity. [a]Values in parentheses represent WHO water quality guidelines (WHO, 2008). [b]Analysis was performed at Cardiff University. MCPP was higher than the drinking water limit, yet concentrations were much lower than reported by other investigators (Baun et al., 2004). m&p-xylene were within range of other published data, typical of MSW leachate (Schrab et al., 1993; Baun et al., 2004). Leachate B3 had fluctuating NH_4^+- N concentrations. According to pH, all leachates exhibited over-lapping acetogenic and methanogenic status.*

Landfill Leachate	Month	PSA		Equivalent H_2O_2 µM	NH_4^+-N mg/L (0.5)[a]	pH	COD mg/L O_2	BOD mg/L O_2	SS mg/L	Cl^- mg/L (250)[a]	MCPP µg/L (10)[a]	m&p xylene µg/L (500)[a]
		TD_{25}	TD_{50}									
C3	August	-	51	-	307	8.2	657	22	478	644	-	-
C1	May	-	53	46	1290	8.0	2580	203	16	1330	-	16.60
C3	March	-	57	14	57.7	8.1	128	10	19	173	-	-
B2	January	-	58	34	114	8.3	-	8	25	336	-	-
C3	May	-	59	10	0.7	8.0	156	20	46	98	-	-
C1	August	-	64	65	1430	7.9	-	-	64	1290	-	-
B2	February	-	70	6	6.5	8.3	-	8	19	338	-	-
B1	August	-	76	20	353	8.5	-	15	44	833	-	-
B1	July	-	81	53	306	8.5	-	20	31	772	21.60	-
B2	June	-	81	-	140	8.4	-	17	20	389	-	-
B2	July	-	81	46	121	8.3	-	13	15	410	10.20	-
B2	August	-	81	58	124	8.3	-	11	40	474	-	-
B2	October	-	82	6	76.6	8.0	-	4	9	206	4.64	-
B2	September	-	83	5	80.4	8.1	-	-	40	243	-	-
C2	June	-	91	-	-	7.4	-	-	-	1500	-	-
B1	February	-	94	2	0.89	8.6	-	17	42	637	-	-

Table 2.2 continued: *Bioreactivity, physical and chemical characteristics of the leachate samples listed in order of decreasing PSA toxicity.* [a]*Values in parentheses represent WHO water quality guidelines (WHO, 2008). Reservoir leachates B3 and C3 presented highest levels of suspended solids. Restored phase leachate B2 had stable Cl^- concentrations, but the lowest Cl concentration was seen in landfill C reservoir (TD_{50}: 59%).*

Landfill Leachate	Month	PSA		Equivalent H_2O_2 µM	NH_4^+ - N mg/L (0.5)[a]	pH	COD mg/L O_2	BOD mg/L O_2	SS mg/L	Cl^- mg/L (250)[a]	MCPP µg/L (10)[a]	m&p xylene µg/L (500)[a]
		TD_{25}	TD_{50}									
C2	March	-	97	-	-	-	-	-	-	-	-	-
A1	January	-	100	70	331	-	-	-	-	580	-	-
B1	October	-	100	-	1.58	8.5	-	9	24	546	15.20	-
C2	August	12	-	-	-	7.2	-	-	-	1388	-	-
B2	April	32	-	-	106	8.3	-	10	28	-	-	-
A2	August	34	-	-	-	7.3	-	-	-	82	-	-
A2	September	39	-	-	-	7.5	-	-	433	-	-	-
B2	March	49	-	19	-	8.4	-	-	7	192	-	-
A2	July	57	-	-	-	7.3	-	-	-	76	-	-
A2	October	84	-	-	-	6.9	-	-	-	78	-	-

Table 2.2 concluded: *Bioreactivity, physical and chemical characteristics of the leachate samples listed in order of decreasing PSA toxicity.* [a]*Values in parentheses represent WHO water quality guidelines (WHO, 2008). Restored phase leachate A2 had comparatively low levels of Cl^-; Due to the provision of physicochemical data from site operators, an incomplete dataset was unavoidable as different sites are subject to varying regulatory requirements.*

Landfill Leachate	Month	PSA TD_{50}	Cd µg/L (0.005)[a]	Ni mg/L (0.025)[a]	Cr mg/L (0.050)[a]	Zn mg/L (5)[a]	Cu mg/L (2)[a]	Fe mg/L (0.200)[a]	Pb mg/L (0.025)[a]
A1	September	2	-	0.120	-	-	-	-	-
A3	February	4	5	-	-	-	-	-	-
B3	February	6	0.23	0.041	0.086	0.051	0.022	0.72	0.008
A1	February	7	6	0.250	1.3	0.35	1.17	6.7	-
C2	January	8	0.01	-	1.25	0.53	5.24	7.9	-
A1	April	8.5	3	0.180	-	-	-	-	-
B3	May	10	0.14	0.007	0.018	0.049	<0.005	0.19	-
A1	June	16	1.00	0.120	-	-	-	-	-
B3	March	17	0.20	0.041	0.056	0.038	0.016	0.37	-
A1	October	20	-	0.092	-	-	-	-	-
B3	June	26	0.13	0.039	0.088	0.058	0.031	0.55	0.031
C3	January	26	1.00	0.040	0.017	0.670	0.024	-	0.012
B3	July	27	0.22	0.058	0.100	0.069	0.027	0.67	0.009
A1	July	28	-	0.130	0.600	0.059	0.009	-	0.010
B3	October	33	0.21	0.034	0.038	0.070	0.027	0.25	0.009
B3	April	35	0.29	0.042	0.068	0.053	-	2.34	-
C2	July	35	-	-	-	-	0.027	-	-
A1	August	41	-	0.120	-	-	-	-	-
C1	January	45	8.00	0.150	0.093	6.410	0.21	-	0.340
B2	November	46	0.12	0.025	0.019	0.010	0.006	3.29	0.010

Table 2.3: *Metal characteristics of the leachate samples assessed by PSA. Data kindly provided by landfill operators. TD_{50} = leachate concentration at which 50% plasmid DNA damage occurs. [a]Values in parentheses represent WHO water quality guidelines (WHO, 2008). The highest Fe and Cu concentrations were seen with restored phase leachate C2 (TD_{50}: 7%). The only Zn excess was seen in collective sump leachate C1 (TD_{50}:45%). All samples exhibited Cd concentrations in range of normal UK landfill leachate (Table 1.2).*

Landfill Leachate	Month	PSA TD_{50}	Cd µg/L (0.005)[a]	Ni mg/L (0.025)[a]	Cr mg/L (0.050)[a]	Zn mg/L (5)[a]	Cu mg/L (2)[a]	Fe mg/L (0.200)[a]	Pb mg/L (0.025)[a]
B3	August	47	0.22	0.060	0.100	0.064	0.025	0.61	0.007
B3	September	47	0.60	0.067	0.096	0.059	0.017	0.60	0.009
B1	November	48	0.23	0.056	0.061	0.025	0.026	0.93	0.015
B1	June	51	0.13	0.041	0.095	0.063	0.030	0.58	0.011
C1	May	53	-	-	0.066	0.063	<0.005	3.58	-
B2	January	58	0.10	0.012	0.011	0.017	0.006	1.38	-
C3	May	59	-	-	-	0.024	<0.005	1.69	-
C1	August	64	-	0.170	0.082	0.049	0.010	2.32	-
B2	February	70	0.10	-	0.013	0.008	-	0.34	-
B1	August	76	0.24	0.069	0.120	0.073	0.026	0.70	0.008
B1	July	81	0.25	0.062	0.110	0.068	0.028	0.70	0.009
B1	September	81	0.60	0.094	0.110	0.067	0.025	0.68	0.010
B2	June	81	-	-	0.018	0.023	0.012	1.06	-
B2	July	81	-	0.015	0.023	0.022	0.007	0.70	-
B2	August	81	0.06	0.018	0.026	-	0.007	0.31	-
B2	October	82	0.08	0.009	0.008	0.032	-	0.93	0.005
B2	September	83	1.00	0.017	0.020	0.049	0.005	0.73	0.011
B1	February	94	0.24	0.045	0.093	0.056	0.023	0.76	0.009
A1	January	100	2.00	0.130	-	-	-	-	-
B1	October	100	0.26	0.044	0.045	0.076	0.031	0.26	0.006

Table 2.3 concluded: *Metal characteristics of the leachate samples assessed by PSA. [a]Values in parentheses represent WHO water quality guidelines (WHO, 2008). Low concentrations of Zn and Cu were observed, similar to methanogenic landfills (Table 1.2). A weakly-reactive leachate, B1 (TD_{50}: 100%), contained much more Cd than other, more reactive leachates. All samples presented lower than average Pb, compared to accepted ranges in the literature (Table 1.2).*

2.3.2 *Plasmid Scission Assay*

In order to compare the free radical-generating potential of various landfill leachates, these were screened by the PSA. A non-linear dose-response model was established for all samples (Figure 2.10); however, not all dose-response sigmoidal curves for all leachates are presented, as trends for all samples were similar.

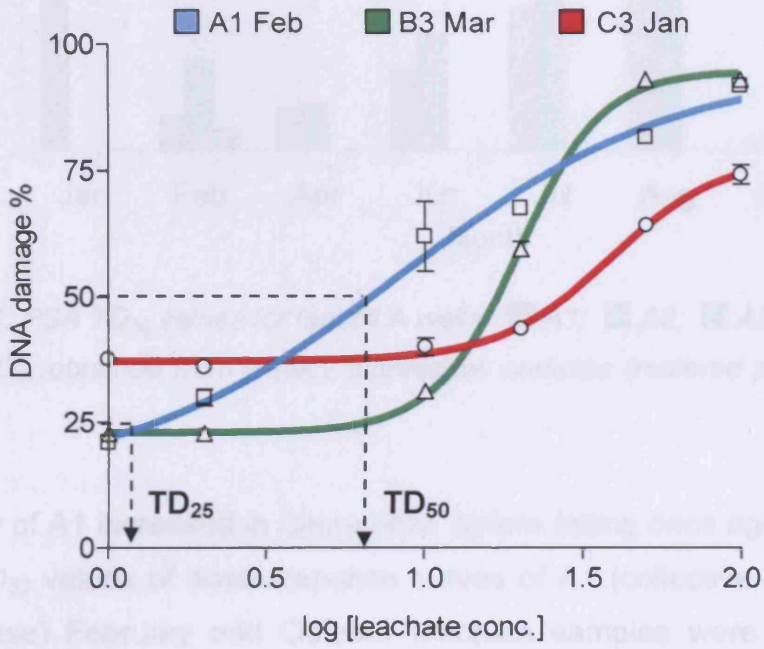

Figure 2.10: *Plasmid DNA damage by diluted south Wales landfill leachates, illustrating extrapolation of TD_{25} and TD_{50} values. The sigmoidal-curves from various leachate samples, irrespective of source, indicate the presence of a threshold response, and sensitivity of the PSA as a model of leachate bioreactivity.*

2.3.2.1 *Landfill A (active and contained site)*

Landfill A (active and contained) winter leachates revealed a significant temporal and spatial variation in bioreactivity, with a general reduction in DNA damage observed over the sampling period (Figure 2.11). The exception to this was seen in the collective sample of active phase leachate, A1, collected during January. This was some of the least damaging leachate collected during the study (TD_{50}: 100%). The subsequent A1 leachate collected in February was significantly increased in bioreactivity (TD_{50}: 7%) before a reduction in damage as summer progressed, to a maximum TD_{50} value of 41% observed in August.

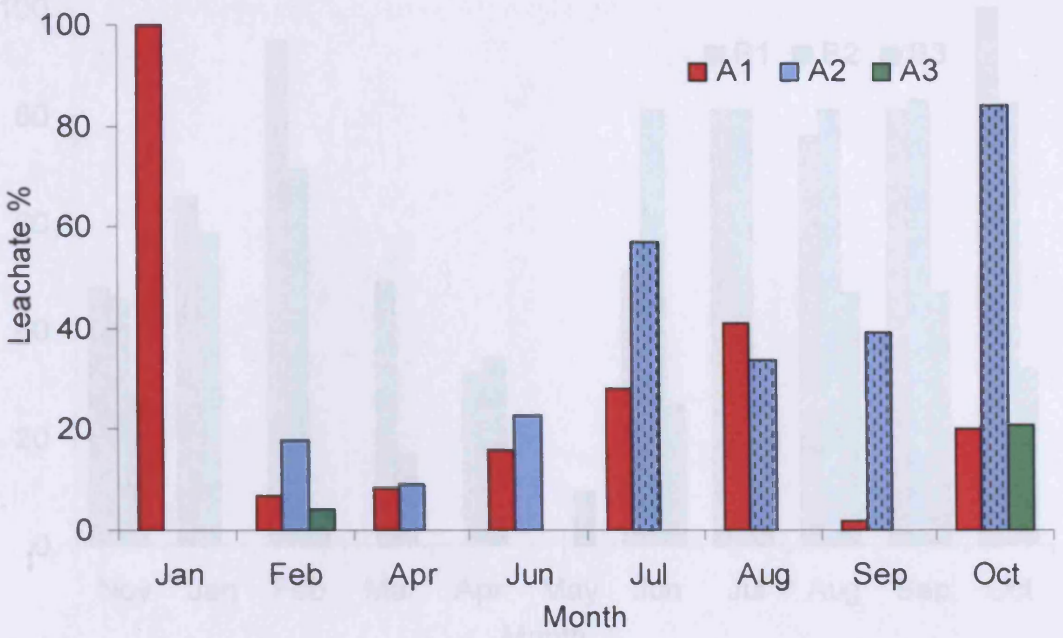

Figure. 2.11: *PSA TD_{50} values for landfill A wells. A1; A2; A3. Lined bars represent TD_{25} obtained from weakly bioreactive samples (restored phase, Jul, Aug, Sep, Oct).*

The activity of A1 increased in September, before falling once again in October. The log EC_{50} values of dose-response curves of A1 (collective sump) and A3 (active phase) February and October leachate samples were compared for similarity by unpaired t-test, which revealed no significant difference between the wells (data not shown).These samples also appeared to be some of the most damaging leachates from landfill A (February TD_{50}: 7% and 4% respectively). Although located in the restored phase of the landfill, A2 exhibited similar bioreactivity to A1 in the spring (April TD_{50}: 9% and 8.5% respectively), and early summer (June TD_{50}: 23% and 16% respectively), before a significant fall in A2 activity ensued, with only TD_{25} values being generated for the remainder of the sampling period.

2.3.2.2 *LANDFILL B (DILUTE AND DISPERSE ACTIVE SITE)*

Landfill B (dilute and disperse, active site) appeared to exhibit distinct spatial variation in PSA toxicity, associated with the different wells during the winter, in the first half of the sampling period (Figure 2.12). Although B1 and B2 followed similar trends during this period, there was a notably similar adverse response

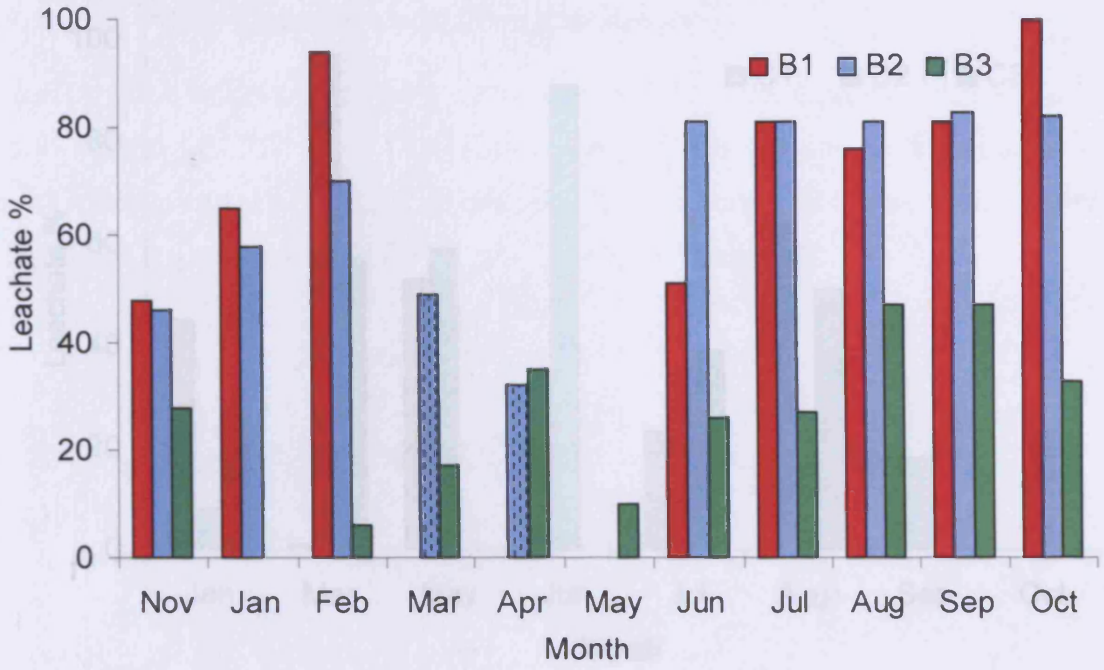

Figure 2.12: *PSA TD_{50} values for landfill B wells. B1; B2; B3. Lined bars indicate TD_{25} obtained (restored phase, Mar, Apr).*

between these two wells' bioreactivity during the later summer months. Overall, the storage reservoir, B3, leachate samples exhibited the greatest bioreactivity, whilst B1 and B2 were less damaging. The most reactive landfill B leachate was obtained from B3 in February (TD_{50}: 6%). A progressive fall in this well's bioreactivity was then observed, to TD_{50}: 35% in April, followed by a period of fluctuating reactivity for the remainder of the study. The most toxic B2 leachate was collected at the start of sampling in November (TD_{50}: 46%), after which a progressive weakening in toxicity was observed, leading to the least toxic B2 samples being collected in March and April, and the generation of TD_{25} values (lined bars in Figure 2.12). Leachate from B1 analysed by PSA also revealed a weakening in bioreactivity from November to February (TD_{50}: 48% to 94% respectively). B1 leachate collected in June was significantly more damaging than that of B2 leachate (TD_{50}: 51% and 81% respectively), but these two leachate samples subsequently yielded similar TD_{50} values until the end of the sampling period.

2.3.3.3 LANDFILL C (RESTORED AND CONTAINED SITE)

Leachates collected from Landfill C (Figure 2.13; restored and contained site)

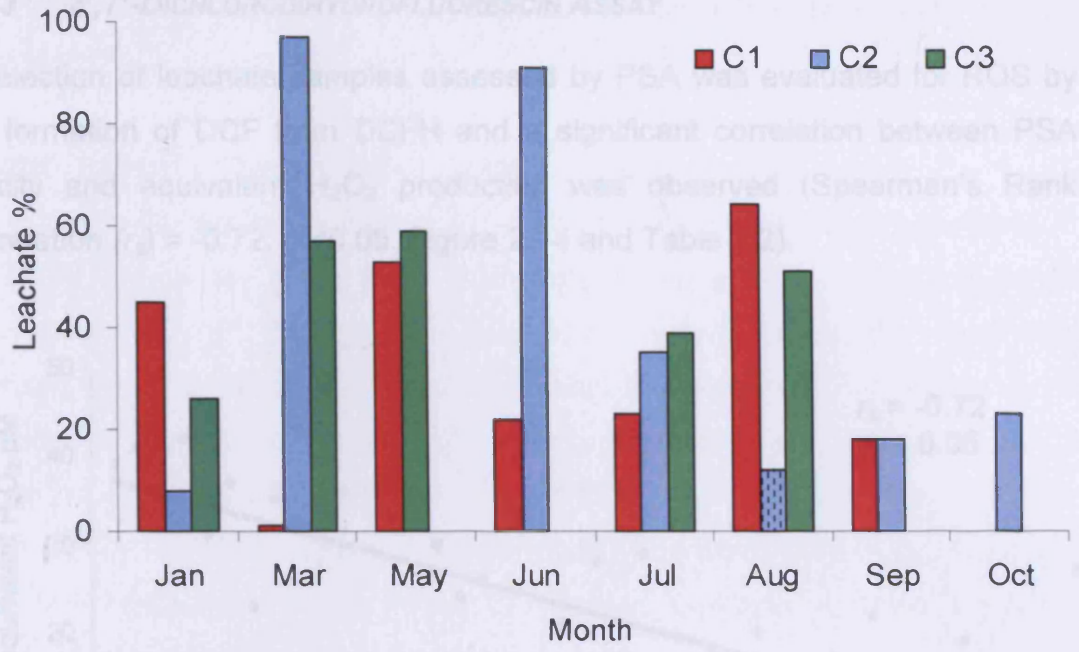

Figure 2.13: *PSA TD_{50} values for landfill C wells. C1; C2; C3. Lined bar indicates TD_{25} obtained (restored phase, Aug).*

exhibited a wide range of bioreactivity. The most free radical-generating landfill C leachate was obtained from well C1 in March, (TD_{50}: 1%), which was the most damaging leachate from any landfill collected during the study. Earlier sampling of C1 in January had demonstrated lower bioreactivity (TD_{50}: 45%), indicating an increase in C1 radical generation from January to March. Seasonal sampling of leachate from this well revealed a varying response of bioreactivity.

The least damaging landfill C leachate was collected from C2, yielding a TD_{25} of 12% in August. In contrast to the seasonal trend elicited by C1 leachate, the TD_{50} of C2 leachate had risen from 8% in January to 97% in March, indicating a decrease in radical generation. However, the fluctuating trend in bioreactivity observed from leachate C1, was mimicked by leachate C2, with DNA-damaging capacity increasing to TD_{50}: 35% in July, before this bioreactivity was reduced in August, culminating in the extrapolation of the only TD_{25} value from landfill C. The effluent collected from C3 yielded TD_{50} values of 26% in January and 57% in March, indicating a fall in reservoir hazard as winter progressed.

2.3.3 2',7'-Dichlorodihydrofluorescin Assay

A selection of leachate samples assessed by PSA was evaluated for ROS by the formation of DCF from DCFH and a significant correlation between PSA toxicity and equivalent H_2O_2 production was observed (Spearman's Rank Correlation (r_s) = -0.72, p <0.05; Figure 2.14 and Table 2.2).

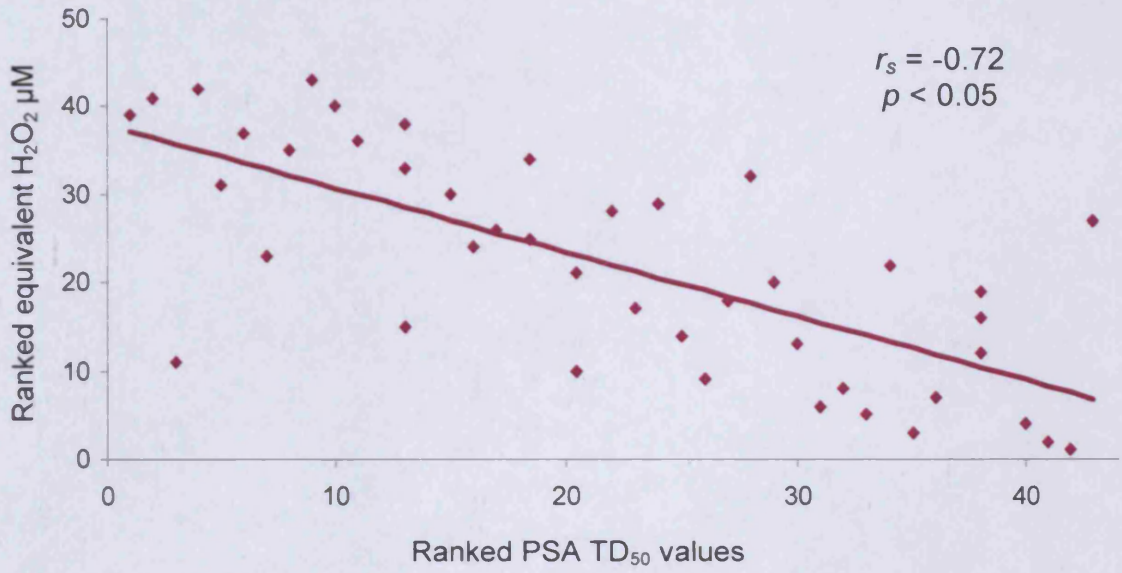

Figure 2.14: *Spearman's Rank Correlation of PSA bireactivity versus equivalent H_2O_2 µM of leachates from various south Wales landfills revealed a reduction in bioreactivity (PSA, x-axis) correlated to decreased free radical generation (DCFH response, y-axis)*

2.3.3.1 Landfill A (active and contained site)

The June and September samples collected from collective sump A1 produced the highest equivalent H_2O_2 compared to all other landfill A samples analysed. The other A1 leachates yielded between 51 and 85µM equivalent H_2O_2, with overall landfill A leachates yielding between 45 and 290µM equivalent H_2O_2. The A2 leachate from the restored phase, produced its highest equivalent H_2O_2 in April, when it was at its most free radical-generating, according to the PSA.

2.3.3.2 Landfill B (dilute and disperse active site)

Landfill B leachates B1 and B2 appeared to exhibit similar ranges of equivalent H_2O_2, with levels existing below 60µM at all time points assessed. In contrast to these two leachates, samples from the leachate reservoir B3 displayed highly

elevated levels of equivalent H_2O_2, with levels reaching approximately 300µM equivalent H_2O_2 in February and May.

2.3.3.3 *LANDFILL C (RESTORED AND CONTAINED SITE)*

Leachates collected from the collective sump C1, exhibited a steady increase in the DCFH response, rising from 43µM equivalent H_2O_2 in January, to 86µM equivalent H_2O_2 in September. The January and September leachates collected from C2 (restored phase), produced the highest DCFH fluorescence intensity compared to the other landfill C leachates, with levels reaching approximately 150µM equivalent H_2O_2; however, leachate collected from the same well in July exhibited a lower response (72µM equivalent H_2O_2). Detected equivalent H_2O_2 levels in C3 (leachate reservoir) samples revealed a gradual decrease from 44µM in January to 10µM in May.

2.3.4 *METAL CHELATION IN PSA AND DCFH ASSAYS*

The physicochemical characteristics (Table 2.4) of the landfill leachate sample selected for chelation experiments revealed the leachate was close to neutral pH, and indicated an acetogenic status, which was also supported by the metal content. The high temperature of the sample was an indication of the active metabolic condition within the waste mass, confirming separation from the atmospheric environment. All physicochemical parameters confirmed an average active phase UK landfill leachate was selected for chelation (Baun and Christensen, 2004).

There was a wide-ranging reduction in the percentage response following leachate chelation (Figure 2.15). The DTPA, EDTA and Chelex treatments caused the most significant suppression of bioreactivity in both PSA and DCFH assays. Although DMG and ABO also caused a significant reduction in bioreactivity in the PSA, the DCFH assay was not sensitive to ABO-treatment. The relationship between the two ROS-sensitive assays was assessed with Pearson correlation analysis and a significant positive association between the PSA and DCFH assay was established ($r = 0.91$, $p < 0.05$).

PARAMETER	VALUE	PARAMETER	VALUE
pH	7.07	P (mg/L)	3
Temperature °C	22.6	Ca (mg/L)	350
Conductivity (µS/cm)	14595	Fe (mg/L)	34
TDS (mg/L)	7310	V (µg/L)	175
ORP (mV)	-179	Cr (µg/L)	914
NH_4^+-N (mg/L)	703	Mn (µg/L)	1750
Cl^- (mg/L)	1052	Ni (µg/L)	1751
SO_4^{2-} (mg/l)	10.7	Cu (µg/L)	469
Na (mg/L)	2477	Zn (µg/L)	984
Mg (mg/L)	263	As (µg/L)	179
Al (mg/L)	16	Cd (µg/L)	4
K (mg/L)	1013	Pb (µg/L)	309

Table 2.4: *Physicochemical properties of the MSW landfill leachate chosen for chelation studies.*

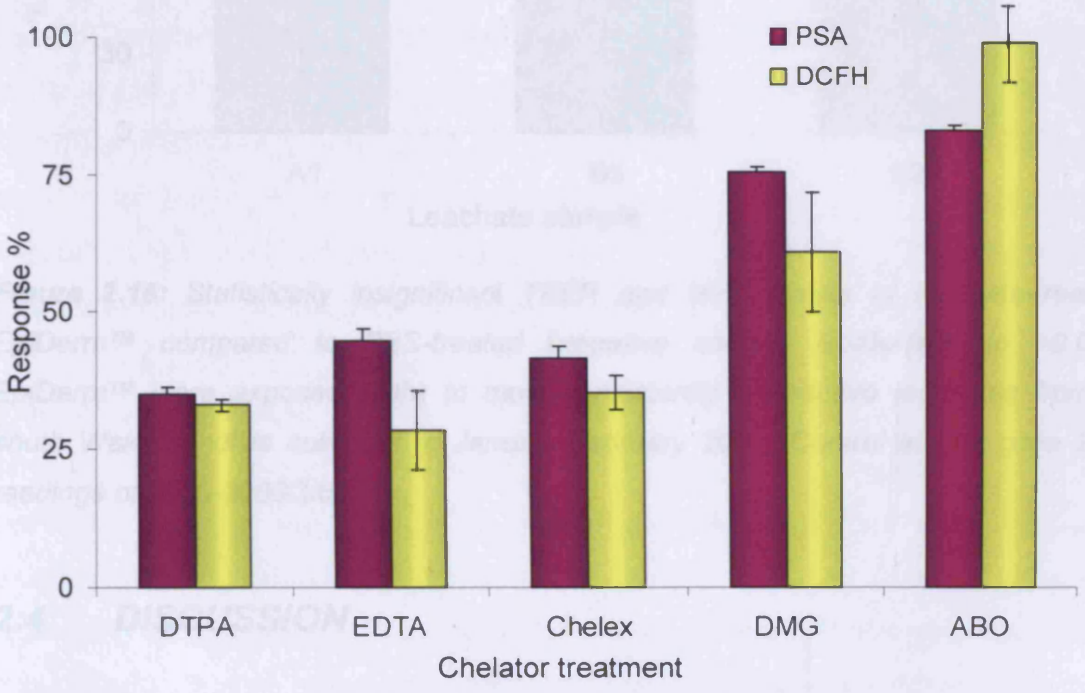

Figure 2.15: *The effect of different metal chelators, on the percentage response of landfill leachates in the PSA and DCFH assays. All treatments caused highly significant attenuation ($p < 0.01$) in both PSA and DCFH systems, except ABO (Normalised data; n=12; mean ± stdev)* ■ *PSA;* ■ *DCFH assay.*

2.3.5 EpiDerm™ Exposure

EpiDerm™ cultures were assessed for tissue integrity pre- and post-leachate exposure. No significantly different TEER values were obtained from tissues exposed to the leachates when compared to PBS (negative control). Post-exposure mitochondrial activity, determined by the MTT assay, did not reveal a statistically significant difference in cell viability in leachate-exposed tissue (Figure 2.16). PBS-exposed EpiDerm™ cultures provided TEER values of 2000-3000Ω/cm^2. Refer to Table 2.2 for physicochemical parameters (A1 February, B3 February and C2 January).

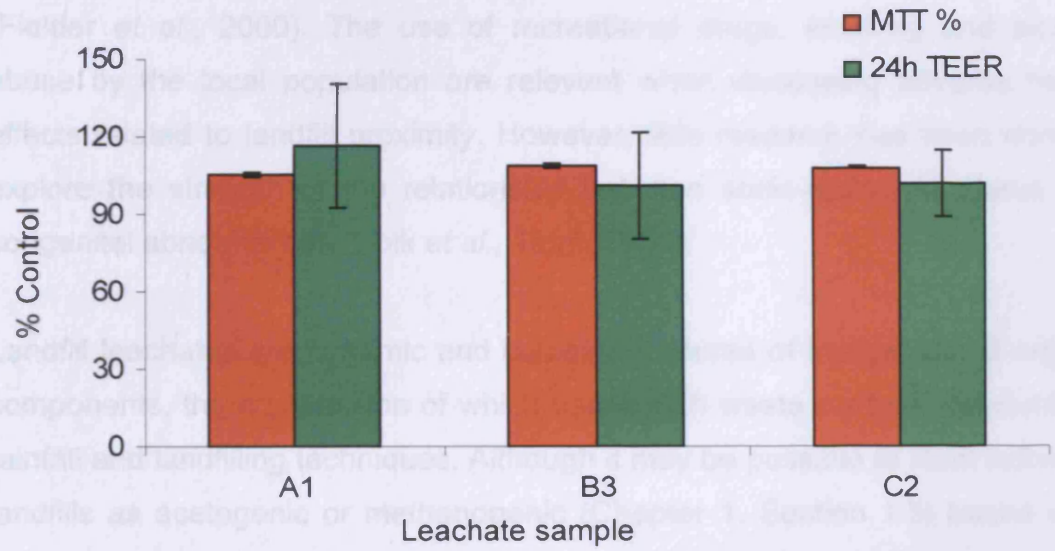

Figure 2.16: *Statistically insignificant TEER and MTT results of leachate-treated EpiDerm™ compared to PBS-treated (negative control) EpiDerm™ (p >0.05). EpiDerm™ were exposed (24h) to most consistently bioreactive leachates from 3 south Wales landfills collected in January-February 2007. Control inserts gave 24h readings of 2000-3000Ω/cm^2.*

2.4 DISCUSSION

High levels of air, water and soil contamination in a few well-publicised cases have led to an increasing number of epidemiological studies being carried out on the health risks posed by landfill sites (Berry and Bove, 1997; Fielder *et al.*, 2000). However, the majority of these studies focused on illegal dumps and hazardous waste sites, with fewer investigations of municipal landfills. A wide

range of adverse health associations have been examined such as cancers, reproductive anomalies, respiratory, neurological and gastrointestinal symptoms (Berger *et al.*, 2000).

In general, epidemiological results revealing low birth weights have been consistently reported (Berry and Bove, 1997; Elliot *et al.*, 2001). Yet, this was often shown to be prevalent prior to site operation, and was also known to be associated with other confounding factors. Parental lifestyles, including maternal smoking and nutritional status during pregnancy, as well as socio-economic status are important to the interpretation of these observations (Fielder *et al.*, 2000). The use of recreational drugs, smoking and alcohol abuse by the local population are relevant when discussing adverse health effects related to landfill proximity. However, little research has been done to explore the strength of the relationship between socio-economic status and congenital abnormalities (Dolk *et al.*, 1998, 2003).

Landfill leachates are dynamic and complex mixtures of inorganic and organic components, the composition of which varies with waste content, degradation, rainfall and landfilling techniques. Although it may be possible to label individual landfills as acetogenic or methanogenic (Chapter 1, Section 1.5) based upon regularly measured bulk parameters such as pH, and gas production ratios, degradation may not progress at the same rate within the waste mass.

The release of landfill leachate into public-access water sources is a current problem facing many countries where un-lined and un-capped sites are common (Bakare *et al.*, 2005). The leachate discharge may migrate, contaminating groundwater aquifers and surface waters. This is of great relevance to nations such as the USA, where domestic abstraction of potable groundwater occurs. Similarly, unregulated waste dumps are commonplace in developing nations, where the domestic and agricultural use of untreated surface waters is standard practice; meaning public health may be adversely affected (Narayana, 2009).

In the case of occupational leachate exposure, landfill workers could be exposed to a mixture of chemicals, which support the study of whole effluent exposure as an indication of the hazard leachate may pose to workers. A study by Gray *et al.* (2005), ranked leachate exposure via dermal contact to pose a greater risk than leachate aerosol ingestion or inhalation of volatile organic compounds released from leachate.

Current environmental risk assessments regarding waste effluents are based on measurements of physicochemical parameters; however, this does not take into account the interaction of complex chemical mixtures with biological systems. In order to determine the potential adverse health effects of landfill effluent, samples were collected from landfills in south Wales operating under different conditions - active, dilute and disperse, containment, restored - and screened for their bioreactivity (Koshy *et al.*, 2007). In addition to initial examination for possible relationships between leachate toxicity and commonly measured physicochemical parameters, the free radical oxidative capacity of leachates was specifically focused on as a biological end-point, since oxidative stress and subsequent damage leads to inflammogenic and carcinogenic health effects.

A well-established, sensitive plasmid DNA scission assay (PSA) was used to detect the oxidative damage caused by landfill leachates, without the use of metabolic activation. The PSA is an indication of the ability of the sample to exert potential oxidative stress, most likely by formation of $^{\bullet}OH$ (Donaldson *et al.*, 1997).

A DCFH assay was also performed to determine whether the damage observed in the PSA correlated with the ability of leachates to produce reactive oxidant species capable of inducing DNA strand breaks. This fluorogenic probe has been widely used as an *in vitro* marker for cellular oxidative stress; the novel use with environmental effluent samples in this study merits further investigation as an indicator of bioreactivity. The effect of metal chelation on the leachate bioreactivity in the PSA and DCFH assay was evaluated. Finally, the

cytotoxicity of leachate was evaluated from the exposure of EpiDerm™ human skin model to leachates from all 3 landfills.

2.4.1 PLASMID SCISSION ASSAY

The use of the PSA in this study was relevant: this demonstrates the ability of the sample to generate ROS, such as the •OH free radical, which is known to have deleterious effects in all cellular forms.

2.4.1.1 CORRELATION OF DNA DAMAGE WITH PHYSICOCHEMISTRY

Although no significant correlation between toxicity measured by the PSA and commonly measured effluent determinants was detected, a significant association between the concentration of MCPP and TD_{50} values was observed after ranking analysis (Table 2.2; r_s = -0.817, p <0.05). Classed as a product of herbicide degradation, MCPP has been found in many MSW leachates and is often used as a tracer in studies of groundwater contaminated by leachate pollution (Kjeldsen *et al.*, 2002). The positive correlation of a chlorinated aromatic compound to radical formation, as detected by the PSA was interesting since MCPP reacts readily with the •OH radical (Topalov, 2000) which is known to be highly DNA-damaging. This may also be an indication of the capacity for auto-oxidation of organic species within the complex chemical mixture posed by whole effluent, which may then potentiate oxidative stress in receptor organisms (Li *et al.*, 2004).

2.4.1.2 LANDFILL A (ACTIVE AND CONTAINED SITE)

The landfill A leachates followed very similar trends in PSA response. Effluent from the lined, active part of the site, A3, is drained into the central sump A1. Comparison of EC_{50} values (concentration of leachate where 50% of its maximal effect was observed) obtained from the PSA of A1 and A3 leachates during February and October revealed similar DNA-damaging capacity, and hence, sampling of A1 (collective sump) was considered to be representative of leachate from the active phase of this waste site.

Effluent from A1 collected in February and April were two of the most reactive

leachate samples collected from any of the three landfill sites in this study. However, the same sampling point had previously yielded leachate with a much lower bioreactivity in January. The evaluation of A1 bioreactivity over the following months, with the observation of a consistent pattern in subsequent A1 TD_{50} values, meant the A1 January TD_{50} value had to be approached with caution. Although A2 (restored phase) also exhibited consistently high reactivity during the initial months sampled, this was postulated to be due to the temporary practice of leachate being pumped from the active phase to the restored phase whilst the site operators were awaiting effluent discharge consent from the regulatory authorities (Hutchings, J., personal communication, 2006). Further sampling of A2 during the remainder of the study appeared to support this hypothesis. The effluent collected from this section of the landfill displayed very weak PSA response, as indicated by generation of TD_{25} values in lieu of TD_{50} values.

2.4.1.3 LANDFILL B (ACTIVE, DILUTE AND DISPERSE SITE)

Landfill B displayed the most distinct well-to-well trends in PSA response, with the greatest bioreactivity exhibited by the leachate reservoir, B3. Although pipe B1 was originally installed to collect surface water, samples collected from it were nearly as damaging as freshly generated leachate collected from B2; the pipeline collecting effluent from the waste mass. This may suggest pipe deterioration or cracking of leachate collection system. It appeared that the hazard of the effluent from B1 and B2 became amplified once in the leachate reservoir B3. Landfill B effluent collected in reservoir B3, before being released to the public sewer system. The reactivity of landfill B leachates in the DCFH assay displayed two distinct responses – a lower range of equivalent H_2O_2 concentrations elicited by both B1 and B2 leachates, whilst the other response was elicited by B3 leachate which generated a higher range of equivalent H_2O_2. These two distinct groups of responses were reflected in results of the PSA, in which leachates B1 and B2 appeared to follow similar trends.

2.4.1.4 LANDFILL C (RESTORED AND CONTAINED SITE)

The reactivity of samples collected from the leachate reservoir C3, varied the least between sampling times. This may be explained by the rapid dilution of

fresh leachate as it mixed into the slow turnover of effluent in this pool. The samples from the restored phase C2 varied in bioreactivity the most, with TD_{50} ranging from 8% in January to a TD_{25} value of 12% in August. Leachate C1, collected from a collective sump, also exhibited a fluctuation in plasmid damage with TD_{50} ranging from 1% in March to 64% in August.

It was concluded that leachate collected from the total waste via sump C1 maintained a higher degree of bioreactivity than C2 leachate. The greater variance seen in the C2 samples may have been due to the conditions within the waste mass proximal to the catchment area of C2, whilst C1 was derived from the total waste mass, which may have shown less variation in composition than specific areas within the landfill. It was noted that C2 leachate was at its least damaging when C1 leachate was also at its least bioreactive, possibly due to dilution by rainfall.

2.4.2 2',7'-Dichlorodihydrofluorescin Assay

2.4.2.1 Correlation of DNA damage with ROS

The fluorescent probe, 2'7'-Dichlorodihydrofluorescin (DCFH), has been widely used as a marker of oxidative stress. Initially thought to be a specific indicator of H_2O_2, DCFH is now considered to be a general indicator of oxidative stress within a system and has been used to detect other ROS and Reactive Nitrogen Species (RNS) such as the hydroxyl radical, ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$), nitric oxide radical ($NO^{\bullet}$) and peroxynitrite ions ($ONOO^{-}$) (LeBel *et al.*, 1992; Gomes *et al.*, 2005). Previous investigators have used this dye to study metals and ROS-based mechanisms of toxicity in cellular and *in vitro* assays (Sugden *et al.*, 2004). In particular, several studies have utilised DCFH in studies upon ambient airborne particulates to estimate the level of oxidative stress within biological systems (Wilson *et al.*, 2002; González-Flecha, 2004; Venkatachari *et al.*, 2005). Consequently, it was decided to use this probe to determine whether the indirect assessment of oxidative stress in landfill leachates was linked to their bioreactivity measured by the PSA.

Detected equivalents of H_2O_2 were used to indicate the presence of ROS within

the leachates, and the significant correlation obtained suggested the presence of ROS in landfill leachates. This supported the findings by previous investigators who have found oxidative damage in murine tissue *in vivo* studies (Li *et al.*, 2005). However, the identity of the components within the leachate samples which caused the oxidative stress within the PSA and DCFH systems remains uncertain.

2.4.3 *Amelioration of Leachate Bioreactivity by Metal Chelators*

The two bioreactivity (PSA, DCFH) assays presented in this study have been previously reported as sensitive indicators of the ROS-activity in particulate and soluble sample studies (Venkatachari *et al.*, 2005; Fan *et al.*, 2006). Although there was no correlation between the physicochemical data provided by the landfill operators and the PSA bioreactivity, the effect of metal-elimination upon the correlation between the PSA and DCFH assays revealed a significant attenuation of leachate bioreactivity, supporting the hypothesis of metal-induced oxidative capacity of MSW leachates. The correlation between the PSA and DCFH assays was made notably stronger following chelation by Chelex, DTPA and EDTA; this may have been due to the mixture of metals acting in synergy, antagonistic or additive ways in Fenton chemistry. These were the chelators with the widest metal specificity. Due to the vast excess of chelator concentration used in the incubations, it was likely that this would have resulted in complete sequestering of free metal species, whilst avoiding a pro-oxidant scenario, as has been highlighted by other investigations (Balcerzyk *et al.*, 2007). The reducing condition of the leachate was likely to have created a favourable environment for metal mobilisation, and since the bioavailability of a metal is dependant upon its solubility, the colloidal fraction of leachate was excluded by filtration from the current investigation.

2.4.4 *EpiDerm™ Exposure*

There is a current paucity of human-specific studies with landfill leachate. Considering that dermal exposure is a potential occupational risk (Gray *et al.*, 2005), and due to the ethics involved, leachate samples from the active phases of all study sites were applied to the human tissue equivalent, EpiDerm™. The

EpiDerm™ model has been used in a variety of *in vitro* studies as a surrogate for animal studies, and a wide range of chemicals have been tested for their irritancy and corrosivity (Netzlaff *et al.*, 2005). EpiDerm™ tissues are metabolically and mitotically alive, unlike harvested cadaver or animal skin models.

The lack of significant difference in tissue integrity (TEER assay) and cell viability (MTT assay) between the control and leachate-exposed EpiDerm™ inserts lends support to the lack of biological effect. The barrier the skin provides has two main functions – prevention of body component loss, and protection against environmental hazards. Intact skin is a robust barrier against penetration by most chemicals and pathogens and also possesses excretory functions. The external layer of the skin consists of a flattened, keratinised layer, which is continuously replaced by cell division and differentiation from the basal layer. The results from this study would seem to indicate that the barrier function of normal human epidermal cells is sufficient protection against landfill leachate absorption and acute toxicity.

2.5 CONCLUSIONS

The PSA and DCFH were found to be sensitive models for the assessment of oxidative capacity of leachates from different types of MSW landfills. Amelioration of bioreactivity by the inclusion of metal chelators supported the the hypothesis of metal-driven oxidative damage. Exposure of EpiDerm™ (human tissue equivalent) to bioreactive leachates did not induce cytotoxicity indicating accidental acute human dermal exposure may be unlikely to result in adverse health effects. However, chronic exposures or repeat acute exposures have not been addressed in this study and must not be excluded from consideration.

According to the PSA, the active and largely uncapped phase of landfill A produced some of the most plasmid DNA-damaging (free radical-generating) leachates, whilst the freshly generated leachate from the unlined and partially

capped landfill B, were some of the least damaging effluent analysed. Yet, the leachate released directly from landfill B leachate storage reservoir into the public sewer system revealed a consistently amplified bioreactivity. Leachate collected from the closed (capped), contained landfill C displayed highly variable bioreactivity profiles, but these results suggested that although the volume of leachate discharged from landfill C was approximately 20% of landfill A, nascent landfill C leachate could be as damaging as leachate generated by landfill A; a currently operating site. The novel use of the oxidant-sensitive probe DCFH in this study suggested the moderate positive association between the amount of DNA damage and the generation of reactive species; this correlation was strengthened by metal chelation, supporting the hypothesis of complex (metal) mixture interactions in potentiating oxidative damage.

Although temporal bioreactivity data was presented in this study, assessment of the seasonal variation of leachate bioreactivity, as measured by PSA and DCFH assays, did not reveal an easily elucidated trend associated with most commonly measured physical parameters such as BOD, COD, SS, pH and metals in this study. Nonetheless, the effective elimination of oxidative stress by metal chelation supported the working hypothesis implicating metals in the potential hazard MSW leachates pose to receptor organisms.

The next chapter will investigate the toxicity of MSW leachate upon *Vibrio fischeri*, in a 'whole-organism' study.

CHAPTER 3

TOXIC RESPONSES OF BACTERIA TO LEACHATE

3.1 INTRODUCTION

The objective of this component of the doctoral research was to compare a 'whole-organism' toxic response to leachate from different types of MSW landfills: active and containment, dilute and disperse, and containment (recently restored) landfills, all located in Wales, UK. Free radical activity, induced by leachates were identified by the *in vitro* PSA, whilst microorganism toxicity was assessed by the aerobic luminescent marine bacteria, *Vibrio fischeri (V. fischeri),* using the commercially available, Rapid On-site Audit Toxicity System™ (ROTAS™).

The marine bacteria, *V. fischeri* (*Photobacterium fischeri*; Girotti *et al.*, 2008), was first suggested as a suitable species for toxicity testing over 20 years ago, and is now used for on-site monitoring of discharges to watercourses in the UK (Johnson *et al.*, 2004; Thomas *et al.*, 2009). This has been attributed to its time scale (usually less than 30min), the ease with which the bioluminescence-inhibition test can be carried out, and the advantage of indicating the toxicity of complex mixtures. *V. fischeri* is a bioluminescent Gram-negative rod-shaped organism, often found in symbiotic association in the light-emitting organs of squid and fish (Stabb, 2005).

Bioluminescence is the process by which a living organism emits photons, and within *V. fischeri* cells, is controlled by the five-gene system *luxCDABE* (Figure 3.1). The two-gene system *luxAB*, codes for the enzyme luciferase. The luminescence is intrinsically linked to the metabolism of the bacteria via the use of NADH and O_2 and the luciferase enzyme system. Luciferase is made up of two protein units, α and β, with the α unit being primarily responsible for the kinetics of bioluminescence (Meighen, 1993). Bacterial luciferase is the catalyst for the oxidation of the corresponding luciferin, reduced flavin mononucleotide $FMNH_2$. In the presence of oxygen and a long-chain aldehyde, $FMNH_2$ is reduced, and emits a blue-green light at 490nm (Meighen, 1993) that is detected in the ROTAS™ luminometer. The products of *luxCDE* are required for (re)generating the aldehyde.

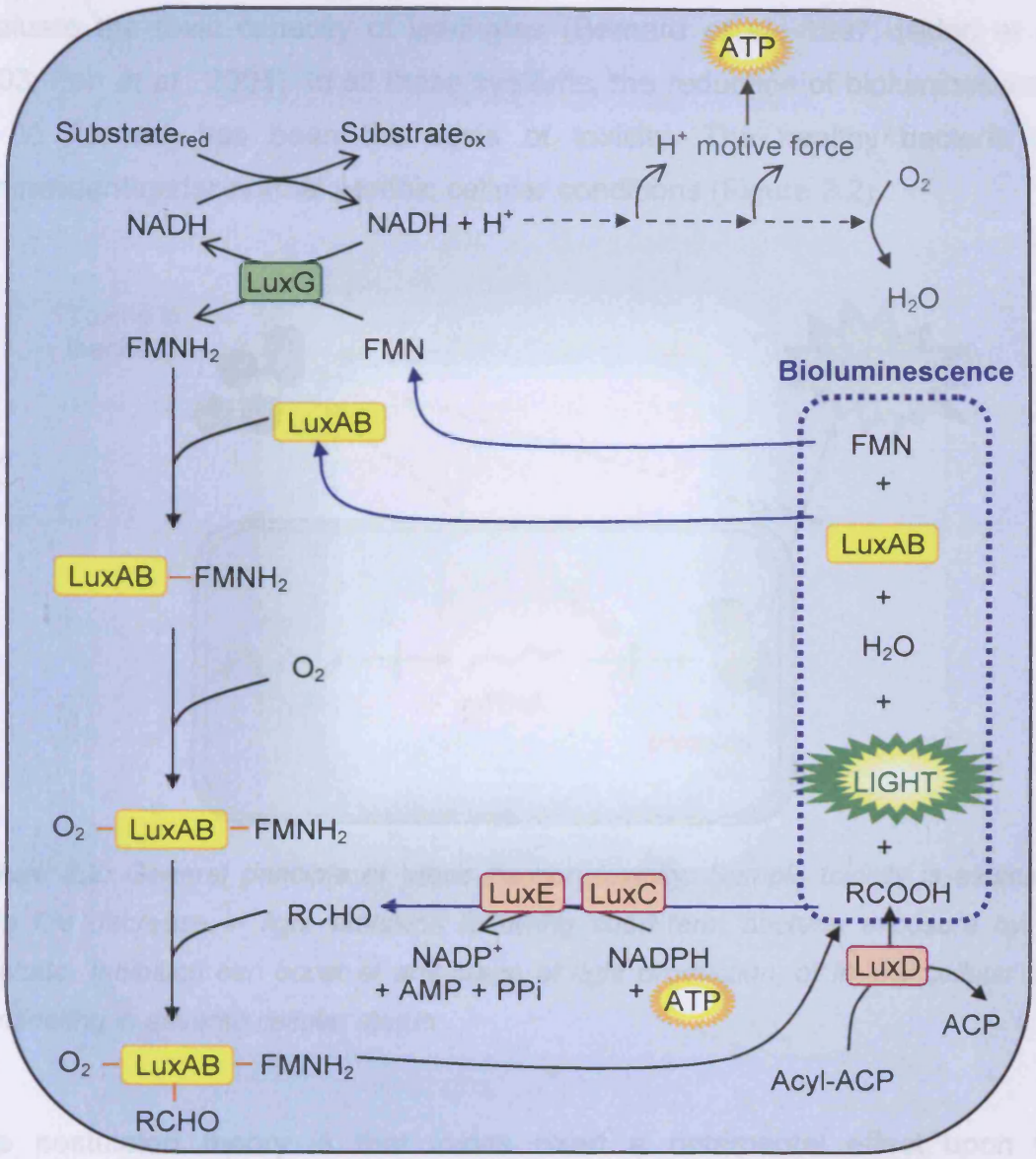

Figure 3.1: *Biochemistry of V. fischeri bioluminescence. LuxG: FMN Reductase; LuxAB: Luciferase; LuxCDE: Fatty Acid Reductase complex. LuxG shuttles $FMNH_2$ reduced equivalents into the bioluminescence reaction. LuxAB oxidises $FMNH_2$ and an aliphatic aldehyde, dissipating the energy as a 490nm photon of light. LuxCDE comprises an acyl transferase, synthetase and reductase which convert fatty acid precursors to the aldehyde required for bioluminescence. This requires ATP and O_2.*

Toxicity tests with *V. fischeri* in commercially available kits such as the ROTAS™, ToxAlert® and Microtox® have been used extensively in environmental monitoring of contaminated soils and aquatic systems, leading to their regular use by national environmental agencies (US EPA, 1993; Gustavson *et al.*, 1998). Previous investigators have utilised these models to

evaluate the toxic capacity of leachates (Bernard *et al.*, 1997; Isidori *et al.*, 2003; Fan *et al.*, 2006). In all these systems, the reduction of bioluminescence by *V. fischeri*, has been the basis of toxicity. The healthy bacteria are luminescent under normal aerobic cellular conditions (Figure 3.2).

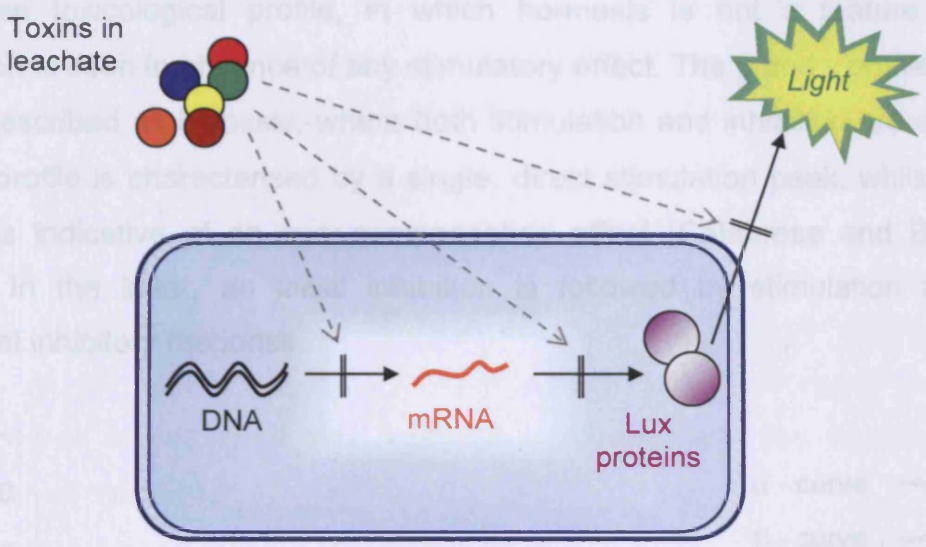

Figure 3.2: *General principle of Vibrio fischeri toxicity. Sample toxicity is estimated from the decrease in light emission following short-term bacteria exposure by the leachate. Inhibition can occur at any stage of light production, or in any cellular site, culminating in adverse cellular status.*

The postulated theory is that toxins exert a detrimental effect upon the metabolism of the bacteria, resulting in a decrease in the level of light produced. However, this means that commercial kits can not take hormesis responses into account. Hormesis has been the subject of a resurgence in interest, with recent reviews by Calabrese and Baldwin (2002) defining hormesis as a "dose-response phenomenon characterised by a low dose stimulation and a high dose-inhibition"; whilst also being defined as an "adaptive response" of the organism to the effects of toxicant exposure (Stebbing, 2000). Although this phenomenon was first observed over a century ago (Christofi *et al.*, 2002), this result is not commonly discussed in ecotoxicological monitoring studies, as the regulatory end-points emphasised are usually lethal or inhibitory effects. Nevertheless, this type of response has

been previously reported by other investigators using *V. fischeri* as an example of sub-acute toxicity (Christofi *et al.*, 2002; Davoren *et al.*, 2005).

A schematic of the three types of response curves obtained by the ROTAS™ assay is provided in Figure 3.3. The α- curve is the typically reported dose-response toxicological profile, in which hormesis is not a feature. Here, inhibition is seen in absence of any stimulatory effect. The β and γ profiles have been described as biphasic, where both stimulation and inhibition are evident. The β-profile is characterised by a single, direct stimulation peak, whilst the γ-curve is indicative of an over-compensation effect (Calabrese and Baldwin, 2002). In the latter, an initial inhibition is followed by stimulation and an eventual inhibitory response.

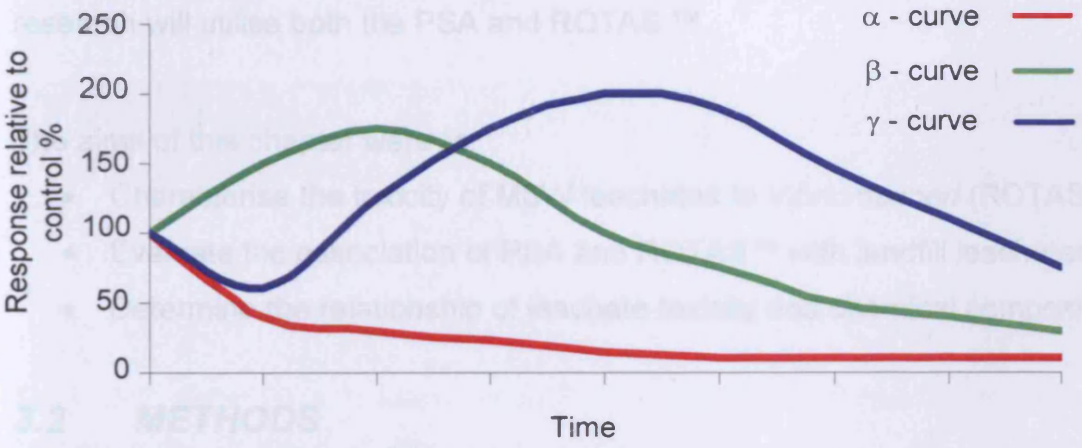

Figure 3.3: *Examples of α-, β- and γ - response profiles. The α-curve is the typically reported dose-response profile. The β-curve is characterised by single stimulatory peak. The γ-curve indicates an over-compensatory response.*

There has been much discussion on the biological advantages of bacterial bioluminescence, since it consumes O_2, reducing equivalents and ATP, which could otherwise be used for biomass production (Bassler and Silverman, 1993). Several researchers have suggested that heightened bioluminescence may stimulate DNA-repair in *V. fischeri*, due to the presence of photolyase (light-dependent DNA-repair enzyme; Christofi *et al.*, 2002; Węgrzyn and Czyż, 2003; Girotti *et al.*, 2008). Hence, agents which damage DNA may also stimulate

bioluminescence (Czyż *et al.*, 2003). However, characterisation of the photolyase by Walker *et al.*, (2006) revealed the process to be energetically inefficient, casting doubt on this hypothesis. Alternatively, by competing for O_2, intracellular O_2-sensitive enzymes may be protected (Stabb, 2005). Recent studies also support an antioxidant-role for bioluminescence. By consumption of reducing equivalents and oxidation of 8 to 16-carbon long aliphatic aldehydes (Nealson and Hastings, 1979), luciferase may confer resistance to oxidative stress by burning excess reductants (Rees *et al.*, 1998; Katsev *et al.*, 2004).

The PSA has been recently used in a previous investigation to assess the ability of landfill leachate to cause DNA damage to a ROS-sensitive plasmid (Koshy *et al.*, 2007). Since oxidative stress and DNA-repair are two hypothesised mechanisms of *V.fischeri* bioluminescence, this section of the research will utilise both the PSA and ROTAS ™.

The aims of this chapter were to:

- Characterise the toxicity of MSW leachates *to Vibrio fischeri* (ROTAS™)
- Evaluate the association of PSA and ROTAS™ with landfill leachates
- Determine the relationship of leachate toxicity and chemical composition.

3.2 METHODS

3.2.1 MATERIALS AND SOURCES All sources are UK-based unless otherwise stated.

Material:	**Source:**
18 MΩ Water	In-house, Cardiff University
Dionex DX-80 IC System	Dionex
Automated sampler ASX- 510	Cetac Technologies, NE, USA
Conductivity and TDS Meter HI-98311	HANNA Instruments
Copper (II) Sulphate	Fisher Scientific
pH, Redox Meter HI-991002	HANNA Instruments
PVDF 0.45µm Filter Discs	Millipore, Cork, Ireland

ROTAS™ Luminometer	Cybersense Biosystems
ROTAS™ Software	Cybersense Biosystems
Saline (0.9% w/v)	Fresenius Kabi
NaCl	Fisher Scientific
Thermo X7 ICP-MS	Thermo Electron Corporation
Vibrio fischeri (1g lyophilised units)	Cybersense Biosystems

3.2.2 SAMPLING METHODOLOGY

All landfill leachates were collected from the collective sump, restored phases, active phase and leachate reservoir (section 2.2.1) within a week spanning January and February 2007. A sample of groundwater collected from an up-gradient aquifer proximal to landfill C was acquired as a model of a clean environmental sample to be processed along with the effluent samples in the PSA and ROTAS™ assays (Table 3.1).

SITE	COLLECTIVE SUMP	RESTORED PHASE	LEACHATE RESERVOIR	GROUNDWATER
A	A1	A2	n/a	n/a
B	B1	B2	B3	n/a
C	C1	C2	C3	C4

Table 3.1: *Sampling well identification from landfill sites of interest. n/a Indicates samples were not collected (well absent or inaccessible).*

3.2.3 PHYSICOCHEMICAL ANALYSIS

Parameters measured at the time of sampling were pH and redox potential (Meter HI-991002), conductivity and TDS (Meter HI-98311). Ion chromatography for Cl^- and SO_4^{2-} was performed at Cardiff University, whilst data for ammoniacal-nitrogen (NH_4^+-N) was provided by the landfill site operators, following analysis by contracted and UKAS-certified laboratories. Analysis of the most ROTAS™-toxic leachate from each landfill, were analysed for metal content by ICP-MS (operation of Thermo-X7 conducted by I. McDonald, Cardiff University).

3.2.4 *Rapid On-site Toxicity Audit System (ROTAS)™*

The ROTAS™ system was purchased from Cybersense Biosystems. The lyophilised organisms were rehydrated in 27ml 2% NaCl for 50min, following which, 1ml aliquots were transferred into a 24-well plate placed in the ROTAS™ luminometer. The system was calibrated to establish basal levels of bacterial luminescence prior to exposure with 1ml undiluted, filtered leachate sample. Preliminary work of the PhD research with the ROTAS™ system, revealed limitations on the validity of the assay due to the temporal restriction on incubation times (i.e. 15min). The duration of the exposure was therefore extended, allowing monitoring of bacterial response to 100min. Readings were collected every minute over a 100min period. Simultaneous incubations of negative controls were incubated with 2% NaCl and positive control wells were exposed to 10mg/L $CuSO_4$. The leachates were between pH 6 - 8 for analysis (n=3), adjusted with 0.1M HCl if necessary. Sample bioluminescence was calculated as a proportion of the saline control.

3.2.5 *Plasmid Scission Assay*

See chapter 2, section 2.2.3 for details of the PSA.

3.2.6 *Statistical Analysis*

Data was exported from the ROTAS™ application software into Excel 2003, where data handling and graphical representation were performed. Statistical analysis included the Anderson-Darling normality test provided in Minitab 14 (Minitab Inc., PA, USA). Further statistical analysis by one-way ANCOVA was carried out with SPSS 14.0 (SPSS Inc., IL, USA), with leachate as the fixed factor, luminescence as the dependent variable and time as a covariate (Fulladosa *et al.*, 2005a). Significance was accepted at $p \leq 0.05$, whilst $p \leq 0.01$ represented high significance and $p \leq 0.001$ represented extreme significance.

3.3 RESULTS

3.3.1 PHYSICOCHEMICAL ANALYSIS

The results of the physicochemical analyses of samples collected from the different landfills reveal varying leachate qualities (Tables 3.2 and 3.3). Leachates A1 and C2 had the highest conductivity levels of 10356µS/cm and 15648µS/cm, respectively. These samples were also the most reducing, and had the highest levels of colloidal inorganic and organic content, indicated by the highest total dissolved solids (TDS) values seen in this study. In contrast, the groundwater sample, C4, had the lowest conductivity and TDS levels (169µS/cm and 84mg/L respectively). Landfill B leachates exhibited little intra-site variation in physicochemical characteristics except for B2 (restored phase), which contained the least amount of SO_4^{2-}. The pH values for all sites ranged from 7.1 - 8.3. Sulphate levels ranged from 3mg/L to 270mg/L in all samples, except leachate C2 (restored phase of inoperative landfill), which contained 4166mg/L SO_4^{2-}.

ICP-MS of the most toxic leachates indicated the majority of metals were within range of standard UK landfill leachate concentrations (Chapter 1, Table 1.2). Nickel was unquantifiable due to instrumental contamination. Leachates A1 and C2 exhibited higher than expected Cr and Cu, whereas leachate B3 contained an excess of Cr only. In general, the major metal elements (Ca, Na K, Mg and Fe) were representative of dilute acetogenic leachates (Table 1.2).

3.3.2 PLASMID SCISSION ASSAY

The majority of samples were assigned TD_{50} values, but TD_{25} values were generated when maximum sample toxicity fell below 50% (Table 3.2). Leachate A1 produced the most free radical activity, as evidenced by the extent of plasmid DNA damage (TD_{50}: 10%). Effluent collected from B2 was the least bioreactive leachate according to the PSA, with a TD_{25} of 100%. Landfill B leachates were generally less DNA-damaging than leachates collected from landfill C, suggesting a relatively weak free radical activity. Leachate C2 was also found to be a relatively weak radical generator (TD_{25}: 20%). The landfill C

Leachate	ROTAS response	PSA		NH_4^+-N	Temp	Cl^-	SO_4^{2-}	pH	TDS	Redox	Conductivity
		TD_{25}	TD_{50}	mg/L	°C	mg/L	mg/L		(mg/L)	Potential (mV)	(µS/cm)
A1	α	-	10	479	22	920	3	7.3	5196	-151	10356
A2	β	-	77	<0.3	10	3	3	7.2	822	48	1638
B1	γ	75	-	153	22	179	202	8.1	3172	53	6360
B2	γ	100	-	191	22	226	92	7.7	2596	74	5200
B3	γ	-	85	167	22	298	270	8.0	2876	66	5656
C1	γ	-	57	780	20	1048	65	7.7	3856	10	7756
C2	α	20	-	-	26	1194	4166	7.2	7736	-391	15648
C3	β	-	75	28	6	436	108	8.3	1957	-14	3974
C4	β	-[a]	-	-	8	13	27	7.5	84	16	169

Table 3.2: *Hormetic responses and physicochemical characteristics of MSW leachates. The α-curve is the typically reported toxic dose-response. The β-curve is characterised by single stimulatory peak. The γ-curve indicates an over-compensatory response. [a] Sample C4 exhibited less than 25% DNA damage in the PSA at any dilution tested, and was classed as a non-reactive sample (Koshy et al., 2008).*

Leachate	Metal (mg/L)											
	Ca	Na	K	Mg	Fe	Mn	Cd	Cr	Cu	Pb	V	Zn
A1 (Collective sump)	143	923	557	109	66	0.763	0.012	1.332	1.169	0.034	0.154	0.353
B3 (Reservoir)	92	491	344	48	67	0.685	0.014	0.934	0.671	0.019	0.189	0.204
C2 (Restored phase)	423	1159	694	315	79	0.509	0.014	1.254	5.236	0.020	0.186	0.532

Table 3.3: *Metal concentrations of leachates which caused greatest reductions in bioluminescence at each landfill site (A1, B3 and C2). Arsenic and Hg were undetectable, B3 exhibited lowest levels of major organic material-derived elements (Ca, Na, K and Mg). Chromium was above standard UK landfill leachate levels in all leachates; Cu was within range for B3 only (refer to Chapter 1, Table 1.2).*

groundwater sample (C4) exhibited the lowest levels of DNA damage and did not fit the non-linear model of the leachate samples. Extrapolation of TD_{25} proved impossible; hence C4 was classed as an unreactive sample.

3.3.3 *ROTAS™*

The extremely significant relationship between time and luminescence was supported by the ANCOVA analysis (p <0.001). The sensitivity of *V. fischeri* was confirmed by the inclusion of a positive control in the form of $CuSO_4$ (10mg/L) in each assay; this also verified the correct implementation of the ROTAS™ protocol. The addition of Cu^{2+} produced a rapid and steep reaction curve and is thought to exert its detrimental effect by inhibiting or competing with bacterial enzyme systems (Madoni *et al.*, 1996).

The majority of samples from all landfills exhibited an extremely significant (p <0.001) stimulatory effect, with greatly elevated bioluminescence observed in seven of the nine leachate incubations.

Of the samples exhibiting biostimulation, β- and γ-type hormesis curves were obtained, with the maximum response ranging from 130% to 245%, compared to the saline controls (Figures 3.4 to 3.6).

3.3.3.1 *ROTAS™ RESPONSE - LANDFILL A*

Landfill A active phase leachate A1 produced an atypical α-curve (Figure 3.4). Although bacterial luminescence decreased to 37% within 1min post-exposure, this was followed by a short recovery period peaking at 25min, before a permanent fall in light output was observed. It was noted that this transient recovery episode did not attain pre-exposure luminescence levels. The restored phase leachate A2 produced a β-response, with luminescence decreasing to sub-control levels at 100min.

3.3.3.2 *ROTAS™ RESPONSE - LANDFILL B*

Landfill B leachates yielded γ-type curves, with all samples undergoing an immediate fall in luminescence within 1min post-exposure (Figure 3.5). This

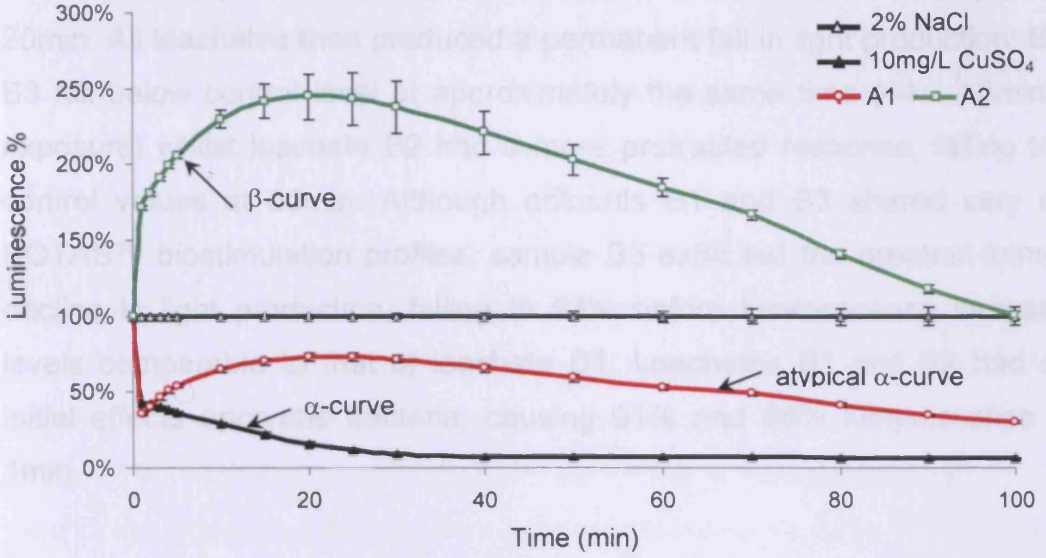

Figure 3.4: *ROTAS™ assay response profiles of landfill A leachates; mean ± SD (n=3). Collective sump leachate A1 (atypical α-curve) produced a very rapid decrease in light production, followed by a transient recovery period. Restored phase leachate A2 ellicited a stimulatory response from the V. fischeri (Koshy et al., 2008).*

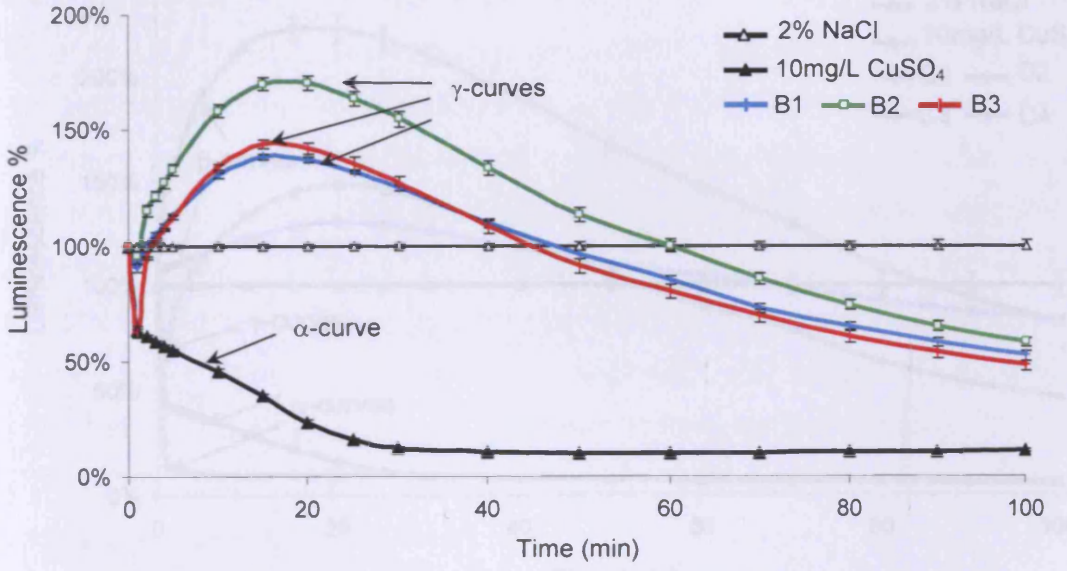

Figure 3.5: *ROTAS™ assay response profiles of landfill B leachates; mean ± SD (n=3). All leachates produced γ-responses, with the reservoir leachate B3 causing the greatest initial decrease in bioluminescence, before an over-compensatory increase in light-production, similar to collective sump leachate, B1. Leachate B2 from the restored phase displayed the highest level of bioluminescence and a protracted decrease to sub-control levels (Koshy et al., 2008).*

was followed by biostimulation above control luminescence levels, peaking by 20min. All leachates then produced a permanent fall in light production; B1 and B3 fell below control level at approximately the same time (44 – 45min post-exposure) whilst leachate B2 had a more protracted response, falling to sub-control values at 60min. Although effluents B1 and B3 shared very similar ROTAS™ biostimulation profiles, sample B3 exhibited the greatest immediate decline in light production, falling to 63% before luminescence increased to levels comparable to that of leachate B1. Leachates B1 and B2 had similar initial effects upon the bacteria, causing 91% and 95% luminescence within 1min.

3.3.3.3 ROTAS™ RESPONSE - LANDFILL C

Landfill C leachates produced the most varied ROTAS™ responses, with α-,β- and γ- curves obtained (Figure 3.6).

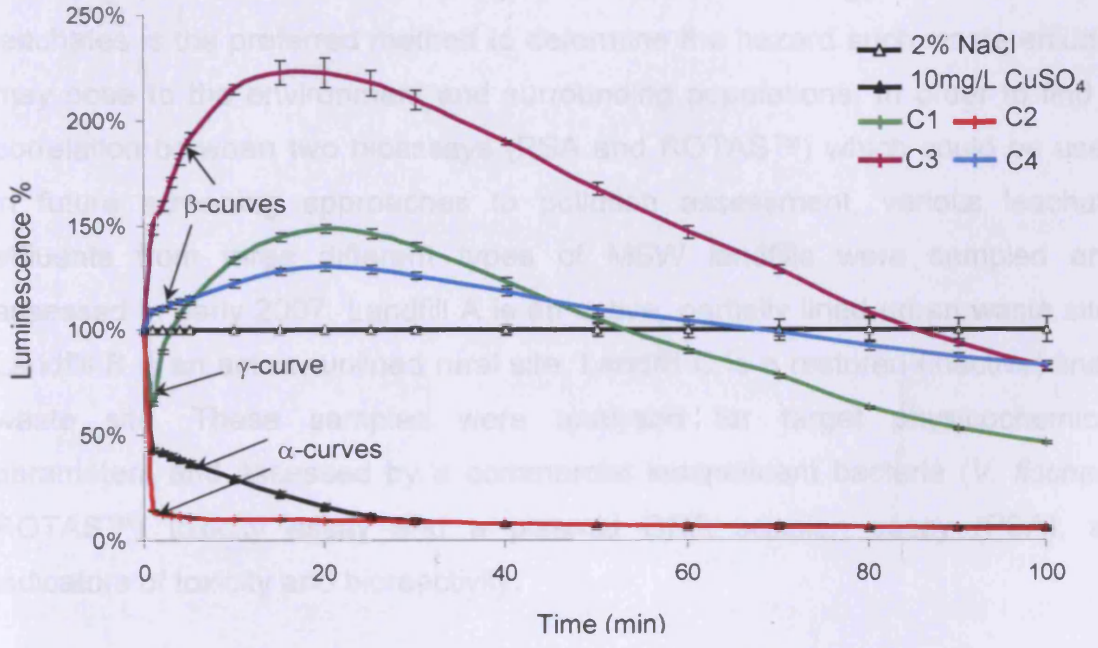

Figure 3.6: *ROTAS™ assay response profiles of landfill C samples; mean ± SD (n=3). Collective sump leachate C1 yielded a γ-curve, and active phase leachate C2 exhibited an α-curve with a steeper gradient than that of the positive control $CuSO_4$. V. fischeri responses to reservoir leachate C3 and groundwater C4 have been classed as β-curves; the later was stimulated to a lesser extent than C3 (Koshy et al., 2008).*

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Collective sump leachate C1 produced a γ-curve, with luminescence falling to 65% within 1min post-exposure. This was followed by biostimulation, peaking at 149% after 20min, before a permanent decrease in luminescence, with light production falling below control levels after 53min incubation. Active phase leachate C2 yielded a typical example of a steep α-type response curve, where luminescence fell to 14% after 1min, and remained below this level for the duration of the assay. Samples C3 (reservoir) and C4 (groundwater) shared similar ROTAS™ profiles; effluent from the reservoir C3 produced a β-curve, peaking at 245% which was more pronounced than that of leachate C4, which peaked at 130%. Effluent C3 also exhibited a prolonged decline in light production compared to C4, with sub-control luminescence thresholds being crossed at 86 and 65min respectively.

3.4 DISCUSSION

An integrated chemical and biological assessment strategy of MSW landfill leachates is the preferred method to determine the hazard such waste effluent may pose to the environment and surrounding populations. In order to find a correlation between two bioassays (PSA and ROTAS™) which could be used in future screening approaches to pollution assessment, various leachate effluents from three different types of MSW landfills were sampled and assessed in early 2007. Landfill A is an active, partially lined urban waste site. Landfill B is an active, unlined rural site. Landfill C is a restored (inactive) lined waste site. These samples were analysed for target physicochemical parameters and assessed by a commercial luminescent bacteria (*V. fischeri;* ROTAS™) toxicity assay and a plasmid DNA scission assay (PSA), as indicators of toxicity and bioreactivity.

The standard ROTAS™ system was designed to reflect the health of the bacterial population, indicated by light emission after 15min exposure to waste effluent. Light emission is a function of the respiratory activity and indicative of the metabolic rate of the organisms. Although other commercial acute toxicity tests with *V. fischeri* are often limited to 15 – 30min duration, pilot experiments

suggested that this incubation period was not feasible, as late-onset adverse effects were masked by biostimulation. Inspection of the luminescence data revealed an early-phase decrease in light production in several samples followed by massive biostimulation. This would ordinarily have been reported as a non-toxic leachate sample. Hence, the initial duration of the ROTAS™ system as 15min acute-toxicity test was extended to 100min to detect sub-acute toxicity of various MSW landfill leachates. This action was supported by the current literature, which reports differences in bacterial viability when exposed to heavy metal species at increasing incubation times (Fulladosa *et al.*, 2005a). Three types of response curves were identified in the ROTAS™ system. The observed ROTAS™ biostimulation could be due to the phenomenon of hormesis, characterised by a biphasic response, which at present, can not be taken into account with the commercial ROTAS™ software (McNeil, H., personal communication, 2006).

Of the three samples that produced β-responses, one of these was the groundwater (C4) sample. The other two samples were collected from the restored phase of landfill A (A2) and the leachate reservoir in landfill C (C3), which had similar results in the PSA (TD_{50}: 77% and 75%, respectively). These displayed an immediate over-compensation stimulation, which manifested as hyper-production of light from the microorganisms, without the initial decrease as was seen within 1min post-exposure to leachates A1 and C2. As time progressed, the levels of luminescence fell below those of background negative control values.

Of the four leachates that displayed γ-responses, it was interesting to note that all landfill B effluent samples fell into this category, as did leachate from the collective sump of landfill C (C1). In this type of response, there was an initial α-response, which was overcome after 1min post-incubation, resulting in a β-response. Physicochemical parameters of landfill B leachates were very similar, with C1 values of TDS and conductivity in a comparable range.

In all cases of leachate hyperstimulation, the observed slope of decreasing

luminescence - after the microorganisms reached their maximum light production - were very similar. Therefore the time at which the *V. fischeri* returned to control levels, was dependent on the extent of biostimulation attained. This hyperstimulation reached a peak within 25min post-exposure to leachate. Groundwater sample C4 may be causing bioluminescence via a different mechanism to that of the leachates, resulting in a different ROTAS™ profile.

The unexpected biostimulation response ranged from 130% for groundwater C4, to 245% for leachate A2. Although the groundwater sample (C4) was collected as an environmental clean water sample, and had a negligible effect upon DNA damage in the PSA, the mild biostimulation response reported in the ROTAS™ system is of interest since it has a bearing on the interpretation of the waste effluent effects upon the *V. fischeri.*

The mechanism(s) and modulator(s) of toxicity underlying the biostimulation of the microorganisms were difficult to interpret based upon the physicochemical parameters collected. In addition to toxicant interactions with cellular enzyme systems, alternate theories of toxicity mechanisms include the physical interaction of the toxin with membrane lipids or proteins, which could result in disruption of integrity and diminished functionality (Sixt *et al.*, 1995). In support of this theory, it has been reported that membrane-bound Cu^{2+} ions possess the ability to catalyse the formation of hydroperoxide free radicals (Grey and Steck, 2001). Toxicants may also be transported into the cell, thereby reacting with cellular components. Other chemicals may cause a disruption of ATP generation, which occurs across the cytoplasmic membrane (Sixt *et al.*, 1995). Since bioluminescence by the bacterium is an energy-consuming reaction (Węgrzyn and Czyż, 2002), uncoupling of oxidative phosporylation by toxicants would deplete cellular ATP which would otherwise have been used in the light reaction.

Landfill leachates are a highly complex chemical mixture of organic and inorganic compounds. Individual components in such mixtures may act in synergy to cause toxicity (Tsiridis *et al.*, 2006). In this study, high levels of TDS,

conductivity and redox potential were the closest indicators of bacterial toxicity, with leachates from the active phases of landfill A (A1) and C (C2) causing α-type responses in the ROTAS™ assay, with no hyperstimulation observed. However, these samples elicited very different levels of damage in the PSA, with C2 being significantly less bioreactive than A1 (TD_{25}: 20% and TD_{50}: 10% respectively). In work by Tsiridis *et al.*, (2006), two-hour *V. fischeri* exposures to binary combinations of the heavy metals - Cu, Zn and Pb - were found to exert synergistic or additive effects. The concentrations of Pb used in their work were 10-fold higher than in this study; nonetheless, Cu and Zn concentrations were comparable.

It was not possible to determine which of these components (redox, TDS or conductivity) was the dominant stressor, but it is likely that a combination of all three would have played a part in whole effluent toxicity of A1 and C2 in the ROTAS™ assay. The highly reducing conditions of leachates A1 and C2 may have caused a deleterious effect upon the aerobic metabolism of the bacteria, inhibiting respiration, and hence bioluminescence. TDS and conductivity are indicators of the ionic strength of the effluent; however, neither metric differentiates amongst ions. Aquatic toxicity of highly ionic effluents have been shown in various species, including *Daphnia*, *Chironomid* (midge) larvae and fish (Chapman *et al.*, 2000; Kennedy *et al.*, 2005; Weber-Scannell *et al.*, 2007). Effluents that exhibit extremes of TDS and conductivity, as seen in leachates A1 and C2, may induce ionic imbalance in the exposed test organism, causing osmotic stress and leading to mortality. Goodfellow *et al.*, (2000) suggested effluents with TDS above 1340mg/L, and conductivity above 2000µS/cm, might cause toxicity in freshwater species. In the case of *V. fischeri,* a marine organism, this threshold may be expected to be higher due to the natural environment that the bacteria colonize. In this study, samples with a TDS value of approximately 4000mg/L and conductivity of approximately 8000µS/cm did not exhibit mortality. The C2 sulphate level of 4166mg/L was much higher than would be expected for standard UK leachate (5 to 1560mg/L; Chapter 1, Table 1.2). This may have been due to the degradation of filter cake within the waste mass of landfill C (Purchon, 2001). Since leachate A1, the only other sample

that generated an α-curve contained just 3mg/L SO_4^{2-}, this species was unlikely to have been a primary influence in the toxicity of these leachates to *V. fischeri*. Due to the restricted number of samples analysed, the data from the metals analysis is of limited use. Similar concentrations of heavy metals such as Cr, Cu and Zn have previously been shown to be toxic in *V.fischeri*. However, these effects are ameliorated by humic acid (Tsiridis *et al.*, 2006), which was not analysed in the samples presented here. Hence, the free metal ion concentrations within the leachates remained unknown.

Several investigators have reported the relatively high sensitivity of *V. fischeri* to ammonia and organics, in comparison to inorganic compounds (Ward *et al.*, 2002; Pivato and Gaspari, 2006). Metal ions such as Cr and Cd have found to be of minimal toxicity to *V. fischeri*, reducing bioluminescence at approximately 100mg/L (Fulladosa *et al.*, 2005a,b). These investigators also reported significantly higher toxicity from Cu, Pb and Zn at less than 1mg/L, which was comparable to the concentrations found in the leachates of interest (A1, C2, B3). Since Cd has been found to bind and adsorb to the exopolysaccharide layer of the outermost cell wall, this could be a method of damage attenuation (MacKen *et al.*, 2009).

3.5 CONCLUSIONS

This investigation into MSW leachate bioreactivity, as measured by an *in vitro* plasmid DNA scission assay, and leachate toxicity gauged by a whole-organism bacterial luminescence assay (ROTAS™), found little correlation between the two systems. The mechanisms underlying the two bioassays are likely to be varied and complex, as indicated by the concomitant lack of association with commonly measured physicochemical parameters. The leachates generally elicited temporal-dependant hormetic responses in the ROTAS™ system, whilst the PSA system indicated a more commonly reported sigmoidal dose-dependant response.

Leachates from currently active MSW landfills display varying levels of bioreactivity and toxicity (Ward *et al.*, 2002; Koshy *et al.*, 2007). Currently operating landfill A exhibited intra-site-specific variation, with the restored phase displaying much lower levels of leachate bioreactivity and toxicity compared to the active phase. Currently operating landfill B displayed closest correlation between bioreactivity and toxicity for all leachates collected. The closed, inoperative landfill C displayed intra-site specific variation, with all analysed effluents displaying different toxic responses in the ROTAS™ and PSA systems.

Environmental monitoring kits have the benefit of being relatively rapid and cost-effective. Nevertheless, the data presented here also exemplify the caution that must be exercised when unpredicted results are obtained as toxicity may be masked by pre-programmed test duration.

CHAPTER 4

COLLECTION AND CHARACTERISATION OF PARTICULATE MATTER

4.1 INTRODUCTION

Landfill airborne particulate matter (PM) is generated by a variety of mechanical, chemical and meteorological processes that include (but are not limited to):

- Movement of heavy dustcarts and site vehicles over dry, unpaved access roads, diesel exhaust fumes and brake emissions from on-site vehicles
- Action of tipping waste at the working face raises plumes of dust, notably on elevated ground, which are exacerbated by windy conditions
- Waste compaction and re-entrainment of previously deposited waste, by bulldozers and crushers
- Stockpiles of bare earth required for daily waste coverage which are susceptible to suspension and dispersion by wind
- Arid and windy conditions which lead to higher levels of entrained PM_{10} in the local atmosphere (Mozzon *et al.*, 1987; Bridges *et al.*, 2000; Fitz and Bumiller, 2000; Lis *et al.*, 2004).

The importance of particle size to toxicity has been highlighted by a number of experimental studies, which have shown the total surface area of particles to be a good indicator of toxicity. Other investigations have correlated particle composition with biological activity (Oberdörster *et al.*, 1995; Brown *et al.*, 2004; Schwarze *et al.*, 2007). Therefore, particle physicochemistry must be characterised in order to evaluate potential toxicity.

Urban PM_{10} is predominantly anthropogenic in source, and current epidemiological evidence suggests a link with adverse health effects (Dockery *et al.*, 1992; Dagher *et al.*, 2005; Seaton *et al.*, 2005; Kampa and Castanas, 2008). As the speciation and toxicity of urban particulates is relatively well investigated, it was important to compare the landfill PM_{10} collections with an urban sample.

The main research aims of this section of the study were to:

- Collect PM_{10} from an active landfill site
- Collect PM_{10} from an urban location
- Characterise landfill PM_{10} in comparison with the urban collection using Field Emission Scanning Electron Microscopy (FESEM) and IA (Image Analysis) for morphological and size distributions.

4.2 METHODS

4.2.1 MATERIALS AND SOURCES

All sources are UK-based unless otherwise stated.

Materials and Equipment:	**Source:**
A&D GR202 Analytical balance	A&D Weighing, CA, USA
C30 Horizontal Elutriator	Westech
Flow Meter	CT Playton
High Volume Cascade Impactor (HVCI)	Rupprecht and Pataschnick, NY, USA
Isopore Polycarbonate Filters (47mm diameter, 0.67µm pore size)	Millipore
Leica Q500IW Imaging Workstation,	Leica Imaging Systems.
Particle Atlas Electronic Edition 2	McCrone Research Institute, IL, USA
Philips XL30-FEG SEM	Philips Electron Optics, Eindhoven, NL
Polyurethane Foam Substrate (PUF; Chemvol)	Thermo Scientific, Ohio, USA
Quorumtech SC500 Sputter Coater (Gold-Palladium)	Quorum Technologies
Screw cap cover (plastic)	Homebase
SEM Aluminium Pin Stub (12.5mm)	Agar Scientific

4.2.2 SAMPLING LOCATIONS

The Cardiff city landfill, Lamby Way, was chosen as the collection site of PM_{10} (Figure 4.1.a). This was based on several logistical factors:

- Sole deposition site for all Cardiff city municipal solid waste, provided a cross-section of wastes which contribute to landfill mass and emissions
- Active disposal landfill (accessible daily between 07:00 – 16:00h)

- Located within Cardiff city limits, 2 miles from residential areas
- Restricted collecting periods (between 08:30 - 15:30h) dictated by landfill safety management
- Short distance to Cardiff University for transportation of all heavy equipment to and from storage, and rapid access to technical advice
- Logistics of sampling at active landfills meant that the collection devices could never be left unattended
- The absence of mains electrical supply meant that a petrol-fuelled generator had to be deployed to provide power to all collection devices.

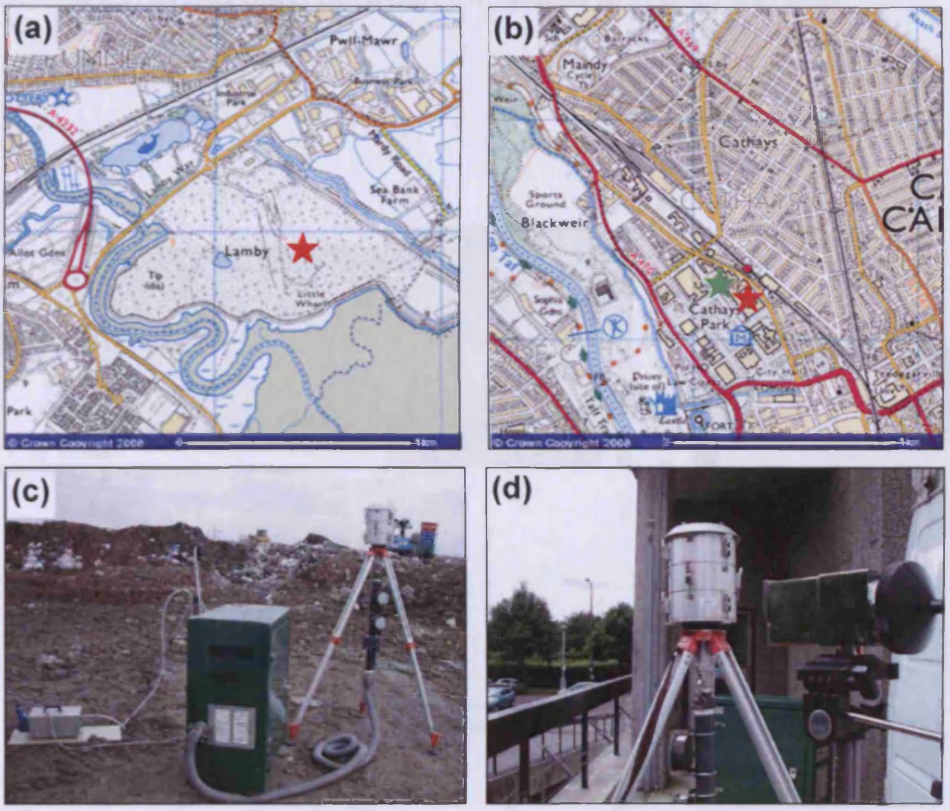

Figure 4.1: *(a) OS® map of Cardiff landfill site, the precise location of the collectors ★ was subject to site operations;* ***(b)*** *OS® map of Cardiff city urban site, location of the collectors 2007 ★, 2008 ★;* ***(c)*** *High Volume Cascade Impactor (HVCI) and Negretti collectors in 2007 landfill sampling;* ***(d)*** *HVCI and Negretti collectors in 2007 urban location.*

Sampling at an urban location, at Cardiff University, approximately 2.5 miles from the landfill was also undertaken (Figure 4.1.b). This strategy enabled comparison of landfill PM_{10} with urban PM_{10}. Two collection devices - Negretti

C30 and High Volume Cascade Impactor (HVCI) - were used in parallel at each site to enable the optimal use of the PM_{10} for the study (Figure 4.1.c, d).

4.2.3 *NEGRETTI C30 SAMPLER*

The Negretti C30 horizontal elutriator (Figure 4.2), controlled by a variable-speed pump, was used to collect PM_{10} onto isopore polycarbonate filters (47mm diameter, 0.67µm pore size, 3.10×10^7 pores/cm^2, 8% void space; Millipore, UK). The cut-off size of the Negretti C30 was a function of air flow velocity, and when set to 30L/min, only particles with an aerodynamic diameter of 10µm or less, would pass between the plates and onto the filter.

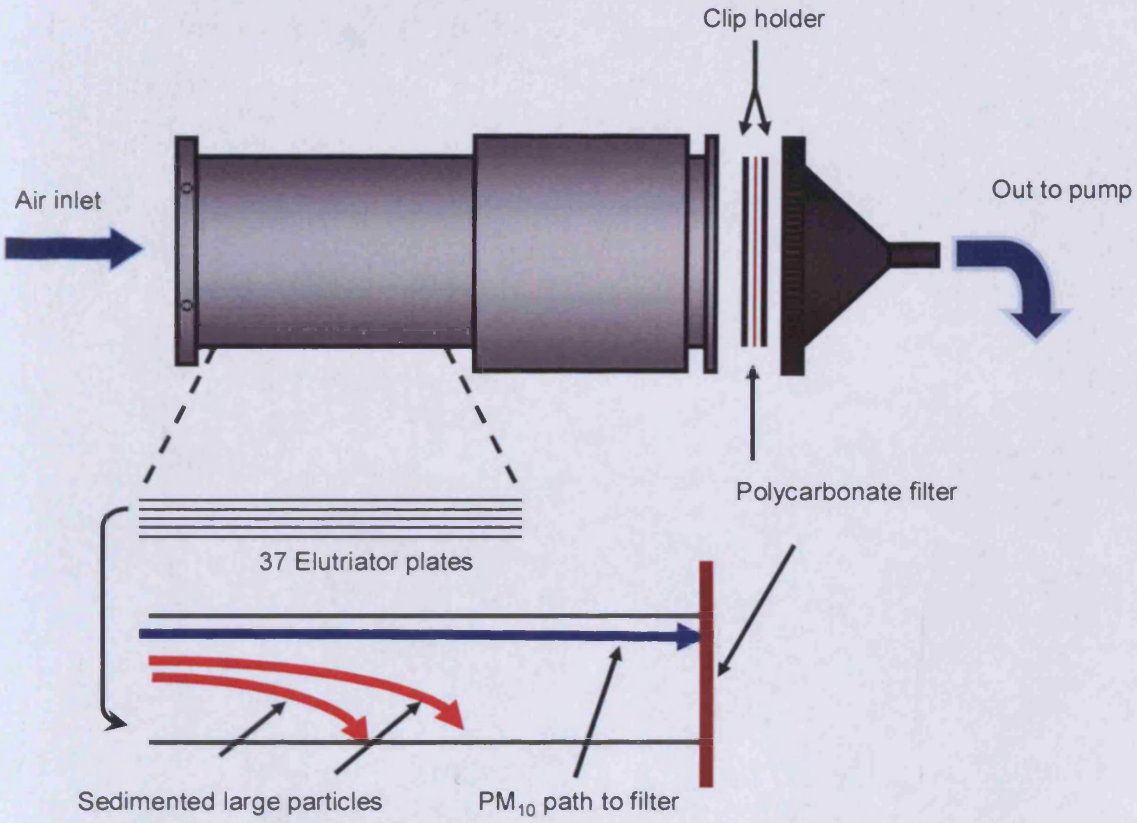

Figure 4.2: *The Negretti C30 elutriator utilised for the 2007 collection of landfill and urban PM_{10} onto polycarbonate filters for characterisation. The head contained 37 horizontal plates enabling size-selective sampling of PM_{10}. The trajectory of PM_{10} is indicated by the blue arrow. Larger particles (> 10µm) impacted onto the elutriator plates, and did not reach the filter.*

The filter was held firmly between two ring clips in the collector head and mounted on a tripod which supported the in-line flow meter (Figures 4.1.c, d). The air was drawn between a series of 37 parallel stainless steel plates, spaced 1.5mm apart, towards the filter at the back of the PM_{10} head. Any particles larger than 10μm would impact and adhere onto the elutriator plates (Greenwell *et al.*, 2002b; BéruBé *et al.*, 2004; Jones *et al.*, 2006).

The Negretti sampler was a portable device as it could be powered by either battery (12V), or mains (240V) supply. Although the Negretti sampler was insufficient for collection of the larger masses of sample required for chemical and toxicological analysis, it was a popular collection system for PM_{10} characterisation. The polycarbonate filters used in the Negretti presented a uniform, inert and stable collection surface, thereby minimising common technical difficulties (movement and charging), under SEM (Jones *et al.*, 2006).

Overloading of the filters was avoided by regular checks of the flow meter – a 5L/min drop in the air flow rate indicates the filter has been fully loaded and must be replaced. This procedure also enabled optimal collection density for bulk PM_{10} particle identification (BéruBé *et al.*, 2006).

4.2.4 High Volume Cascade Impactor

PM_{10} collections for wet chemical and toxicological characterisation were performed in 2007 and 2008, using a HVCI (Rupprecht and Pataschnick, USA), first described by Demokritou *et al.*, (2002) (Figure 4.1.c, d; Figure 4.3). The HVCI consisted of a 3-stage metal cylindrical head, mounted on a heavy-duty tripod (2007; System I) or in a wire-frame cage (2008; System II) and a pump. Air was drawn though the head at 900L/min (System I), or 1100L/min (System II; Jones *et al.*, 2006).

Each of the 3 stages consisted of an acceleration slit and substrate platform beneath it. Inert, metal-free, Polyurethane Foam strips (PUF) were used as the collection substrate. The air flow was pulled under a rain cap and into the stacked collection head, where it was sequentially forced through narrowing

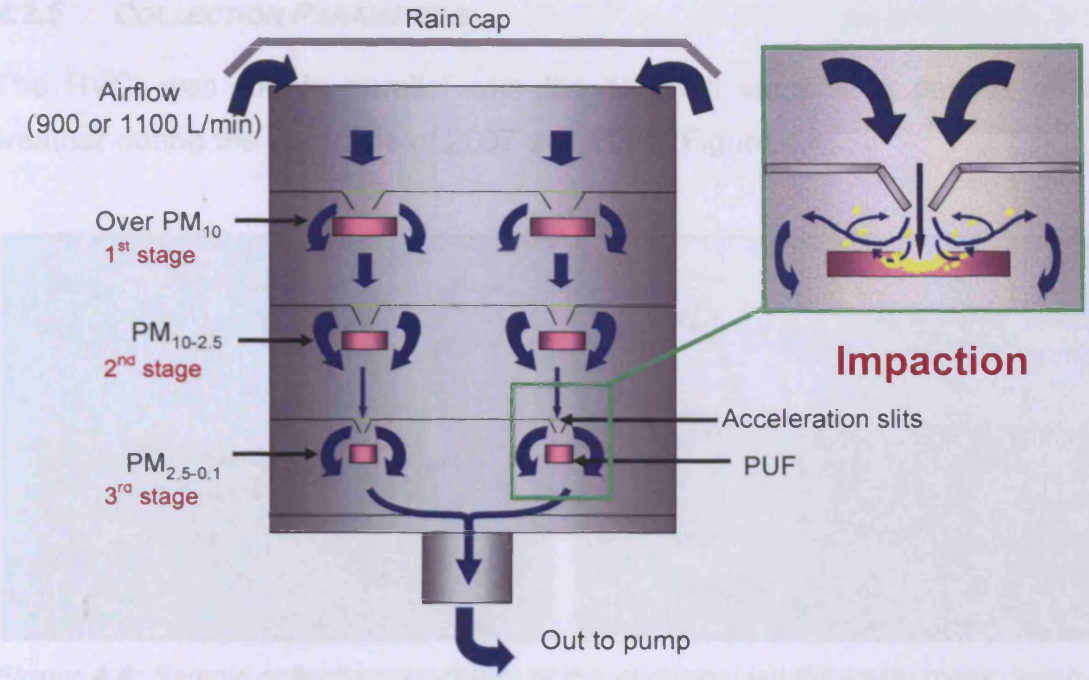

Figure 4.3: *The HVCI utilised for landfill and urban $PM_{2.5-0.1}$ and $PM_{10-2.5}$ collection onto PUF substrates for physicochemical characterisation and toxicological analysis.*

acceleration slits before impaction onto PUF substrates, supported in solid platforms. The HVCI was modified 'in-house' at Cardiff University (a silicone grease-trap at stage 1 was removed to avoid downstream contamination) by the use of a 32mm wide PUF strip (Moreno *et al.*, 2003). This acted as a 'guard' section, blocking the progression of large PM (> 10µm) from the following two sample collection stages. The HVCI operated as a 3-stage PUF collector head, with the two lower stages dividing the PM_{10} into $PM_{10-2.5}$ and $PM_{2.5-0.1}$. The air stream was forced onto the guard PUF, where large PM, such as insects and feathers were retained. The redirected air stream was then bounced-off, and pulled underneath the guard PUF-support platform, through the narrower acceleration slits of the 2nd stage, and onto a 13mm wide PUF substrate that accumulated $PM_{10-2.5}$. The redirection process of the air stream was repeated, and the air flow was directed through the narrowest acceleration slit of the HVCI, and into the 3rd and final stage which collected $PM_{2.5-0.1}$ on a 6mm wide PUF. The HVCI has previously been shown to operate at very efficient size-selectivity (efficiency ≥ 97% for either $PM_{10-2.5}$ or $PM_{2.5-0.1}$ stages; Demokritou *et al.*, 2002); this was confirmed experimentally by previous workers in the research group (Moreno *et al.*, 2003, 2004b; Jones *et al.*, 2006).

4.2.5 COLLECTION PARAMETERS

The HVCI was run in parallel with the Negretti sampler in periods of dry weather during the summers of 2007 and 2008 (Figure 4.4).

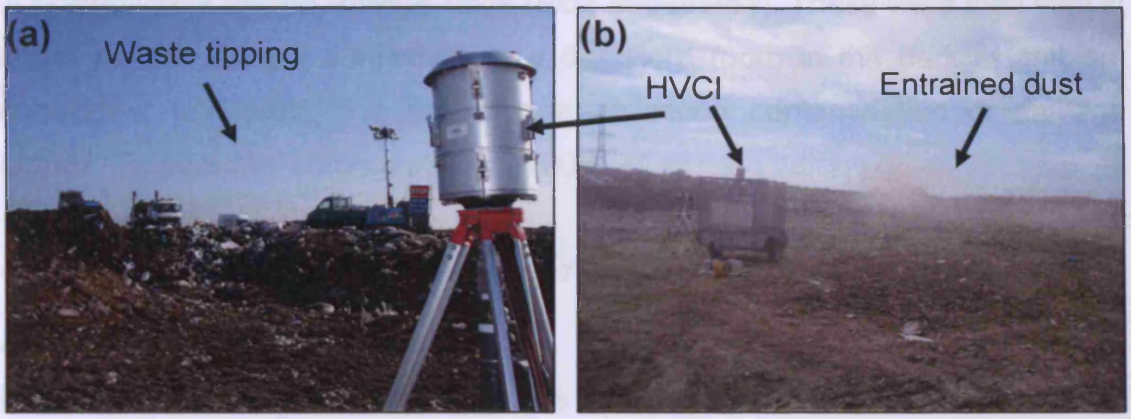

Figure 4.4: *Sample collection conditions at the municipal landfill waste mass during* ***(a)*** *proximity to active waste tipping face (2007) and* ***(b)*** *anthropogenic activity visibly suspending soil-derived PM (2008).*

A requirement of dry ground conditions was deemed necessary for a successful sampling regime, with a period of 48h of dry weather preceding sample collection, since sodden ground conditions would have prevented the transport of the HVCI onto the waste mass. Details of the PM_{10} collection conditions for the samples of study interest were obtained from the Met Office (http://www.metoffice.gov.uk).

The daily activities of the landfill operations had to be ascertained from the site management prior to assembling the sampling devices. The precise locations of the samplers were subject to daily variation, dependent upon the prevailing wind conditions and the respective location of the waste tipping face. However, the Negretti and HVCI samplers were within 5 metres of each other during any PM_{10} collection.

4.2.6 GRAVIMETRIC ANALYSIS

All Negretti filters and PUF substrates were weighed in 5 replicates, pre- and post-sampling, on an analytical balance (A&D GR202, CA, USA) to a sensitivity

of ± 10 µg (BéruBé *et al.*, 1999; Jones *et al.*, 2006). All Negretti filters were replaced daily during each sampling campaign, whilst landfill PUF substrates were replaced after use on a minimum of 3 sampling occasions (Moreno *et al.*, 2004a). All samples were stored in either sterile petri dishes (Negretti filters) or in polyethylene sealed 'ziplock' bags (PUF substrates). These were then kept in larger plastic air-tight containers in a dry store room in the dark at ambient conditions, minimising the risk of post-collection contamination or damage (Jones, T., personal communication, 2009).

4.2.7 *Field Emission Scanning Electron Microscopy*

4.2.7.1 *Sample Preparation*

Negretti samples were morphologically analysed by FESEM in 2007. Direct epoxy resin mounting of the polycarbonate filters onto an aluminium SEM pin stub was not possible, as the adhesive would have been drawn up via capillary action through the filter pores, thereby engulfing any PM_{10} on the collection side of the filter. Instead, filter mounting involved assembling a raised 'drum' on the SEM pin, by gluing (epoxy resin) a hollow plastic screw cap (4mm internal diameter, 12mm external diameter) onto the surface of the pin stub. Epoxy resin was used to mount triangular filter sections upon the screw cap, and to create a bridge from the filter surface to the pin stub (Figure 4.5), thereby preventing 'charging' under the SEM beam. The stubs were allowed to dry for 24h before they were coated for electrical conductance with a 20nm layer of gold-palladium (AuPd). Duplicate coated stubs were prepared for each Negretti filter collected, and a grid-system was used to ensure non-bias of the imaged filter areas (Jones *et al.*, 2006).

HVCI samples were morphologically analysed by FESEM in 2008. Four PUF sections (0.5 -1cm wide) from each collection mode, were bisected to minimise the SEM working distance, and mounted directly onto the surface of the SEM pin stub with epoxy resin (Figure 4.6). These were coated in triplicate with AuPd (as above).

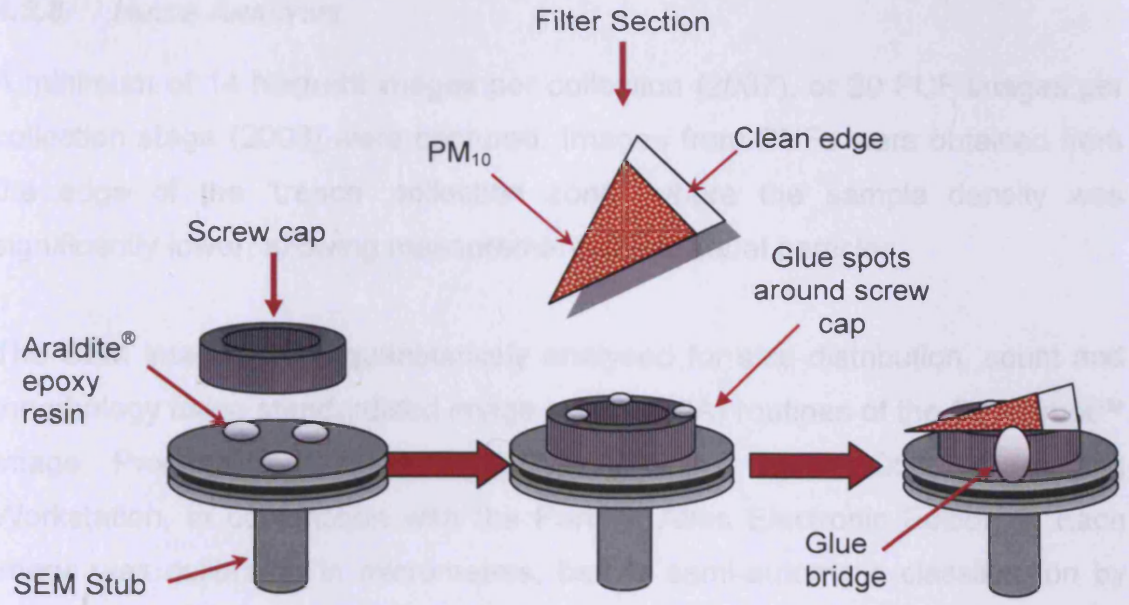

Figure 4.5: *Negretti filter stub preparation for FESEM illustrating 'drum' assemblage.*

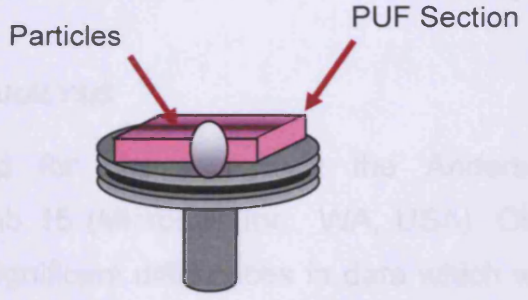

Figure 4.6: *HVCI substrate stub prepared for FESEM. PUF sections were bisected before epoxy resin was used to mount the PUF directly and to link the PUF surface to pin stub.*

4.2.7.2 *FESEM Operation Parameters*

A Philips XL30 FEG Environmental Scanning Electron Microscope was used. It was operated in secondary electron mode, accelerating voltage 20kV, working distance 10mm, 5000x magnification, with a spot size of 4. These operational parameters allowed the capture of high resolution *.tiff files for morphological analysis. An 8-place multi-stub holder was used in order to maximise sample capacity, thereby minimizing vacuum changes and disruption to the sample chamber.

4.2.8 *IMAGE ANALYSIS*

A minimum of 14 Negretti images per collection (2007), or 20 PUF images per collection stage (2008) were captured. Images from PUFs were obtained from the edge of the 'trench' collection zone, where the sample density was significantly lower, allowing measurement of individual particles.

The SEM images were quantitatively analysed for size distribution, count and morphology using standardised image analysis (IA) routines of the Quantimet™ Image Processing Software (QUIPS) on the Leica Q500IW Imaging Workstation, in conjunction with the Particle Atlas Electronic Edition 2. Each image was calibrated in micrometres, before semi-automatic classification by morphological characteristics. The QUIPS software calculated the surface area and generated the equivalent spherical diameter (ESD) of each particle. The PM number counts and ESD data were exported to MS Excel 2003 for further analysis (BéruBé *et al.*, 1999, 2006; Whittaker *et al.*, 2003).

4.2.9 *STATISTICAL ANALYSIS*

Data were assessed for normality with the Anderson-Darling test for homogeneity in Minitab 15 (Microsoft Inc., WA, USA). One-way ANOVA was used to discern any significant differences in data which were parametric. The Mann-Whitney test was used to investigate data which were proven to be non-parametric (SPSS 14.0, SPSS Inc. IL, USA). Size distributions were displayed by producing frequency percentage histograms, with bin ranges ranging from <0.1µm to >10µm (Microsoft Office, 2003). Significance was accepted at $p \leq 0.05$.

4.3 *RESULTS*

4.3.1 *SAMPLING PARAMETERS*

Meteorological data indicated moderate and comparable wind speeds at both sampling locations (Table 4.1). The logistics of the sampling campaign were a

Year	Location	Wind (mph)	HVCI	Date	Start	Finish	Total Hours
2007	Landfill	Southeast (6)	I	19/10/07	10:10	14:30	14
		East (11)		23/10/07	10:10	14:30	
				24/10/07	09:30	14:30	
	Urban	Southwest (5)	I	21/07/07	10:30	17:30	41
		Southwest (7)		22/07/07	13:00	19:25	
		Southwest (6)		24/07/07	09:30	12:20	
		Northwest (6)		08/08/07	10:15	16:45	
		West (6)		09/08/07	10:00	16:45	
		West (11)		10/08/07	10:00	15:00	
2008	Landfill	Southwest (8)	II	10/06/08	09:30	14:30	28
		South (14)		01/07/08	09:25	14:25	
		Northwest (13)		21/07/08	10:00	14:10	
		Northwest (7)		22/07/08	09:40	14:30	
		East (16)		24/07/08	09:30	14:25	
		Northwest (9)		28/07/08	10:10	14:30	
	Urban	East (13)	II	14/05/08	11:20	21:20	182
		East (4)		17/05/08	13:10	-	
		Southwest (9)		21/05/08	-	09:00	
		Southwest (8)		06/06/08	11:00	-	
		Northwest (12)		09/06/08	-	15:30	
		Northwest (9)		30/06/08	09:30	14:00	

Table 4.1: *Sample collection parameters for PM_{10} sampling regimes at landfill and urban sites. HVCI= High Volume Cascade Impactor; Systems I and II. Extended sampling hours were required for urban PM_{10} collections due to the low $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ masses obtained by the HVCI.*

compromise between the requirement for landfill PM_{10} and corresponding urban PM_{10} samples collected with the same equipment. Whilst it was not possible to collect for more than 5.5 consecutive hours on any given landfill sampling day, due to site inaccessibility, total sampling hours of 14 - 28h were obtained for the landfill collections. Urban PUF samples were collected over extended periods, as interim weight checks revealed less sample mass was being obtained (Table 4.2). The urban site was much more accessible than the landfill and total

sampling hours of 41 – 183h were possible; the HVCI was run continuously at this location.

4.3.2 GRAVIMETRIC ANALYSIS

PUF weights obtained from the HVCI sampler indicated a much lower landfill PM_{10} mass concentration in 2007 compared to the collection of 2008. The ratios of $PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ were also calculated, as a higher ratio is indicative of a more respirable PM_{10} population (Table 4.2).

YEAR	LOCATION	$PM_{2.5\text{-}0.1}$ (mg)	$PM_{10\text{-}2.5}$ (mg)	PM_{10} (mg)	MASS CONCENTRATION PM_{10} ($\mu g/m^3$)		$PM_{2.5\text{-}0.1}$: $PM_{10\text{-}2.5}$ RATIO (HVCI)
					HVCI	NEGRETTI	
2007	Landfill	13.42	18.35	31.77	42	30	0.73
	Urban	8.88	19.85	28.73	13	4	0.45
2008	Landfill	30.41	178.57	208.98	112	n/a	0.16
	Urban	36.90	25.17	62.07	5	n/a	1.46

Table 4.2: *PM_{10} masses, airborne PM_{10} mass concentrations and $PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ ratios, indicating particulate pollution levels during sampling campaigns. n/a no sample available due to discontinued use of Negretti samplers in 2008.*

There were significant intra- and inter-site differences in mass concentrations, highlighted by the $PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ ratios. Landfill and urban collections yielded very different ratios in 2007 compared to 2008. The landfill 2007 sample had a significantly larger proportion of fine PM ($PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ ratio 0.73) than the urban collection ($PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ ratio 0.45). However, in 2008, the urban sample had a much larger fraction of fine PM ($PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ ratio 1.46) in comparison to the landfill collection of the same year ($PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ ratio 0.16). The urban 2008 collection exhibited the greatest proportion of $PM_{2.5\text{-}0.1}$ in comparison with any other sample. Conversely, the landfill 2008 campaign yielded the lowest proportion of $PM_{2.5\text{-}0.1}$ obtained in the study. Average mass concentrations were also calculated from Negretti filters in 2007, and were lower than the equivalent HVCI values.

4.3.3 PARTICLE SIZING, MORPHOLOGY AND COMPOSITION

Examples of the microstructure of control and exposed polycarbonate filter and polyurethane substrate sections are presented in Figure 4.7.

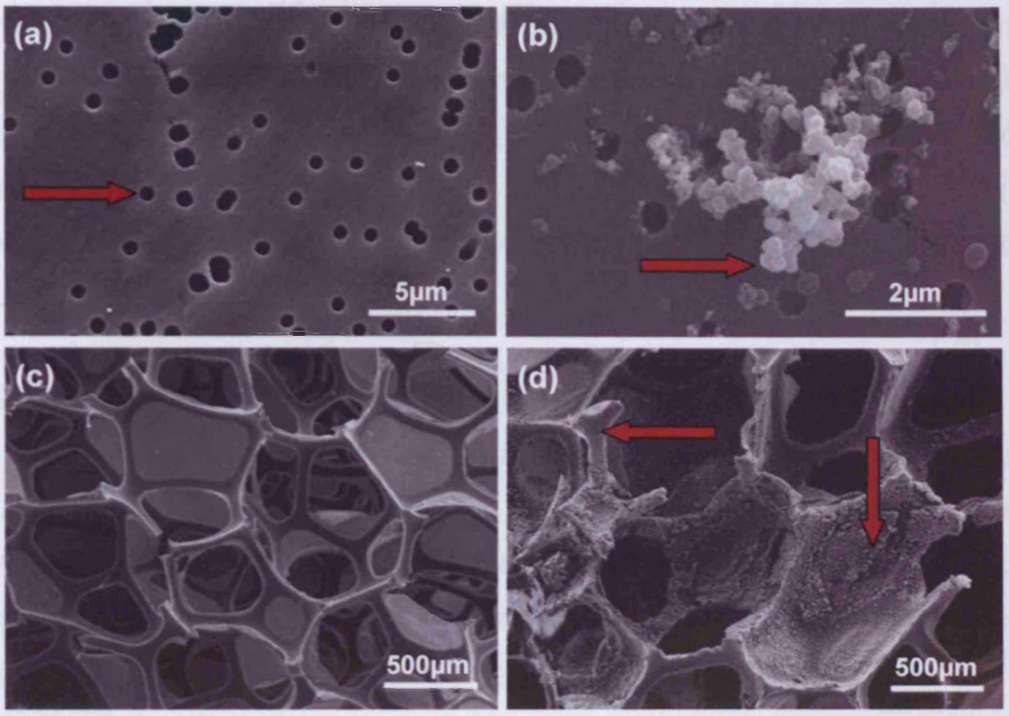

Figure 4.7: ***(a)*** *Unexposed control polycarbonate filter - arrow indicates 0.67µm pore;* ***(b)*** *exposed PM_{10} polycarbonate filter; arrow indicates soot agglomerate chains; note the increased diameter of spherules on the top of this cluster;* ***(c)*** *Unexposed PUF honeycomb 3-D microstructure;* ***(d)*** *Exposed PUF - arrows indicate the saturated PM_{10} "trench", and peripheral area at which photomicrograph images were captured for IA in 2008.*

The smooth and flat planar surface of the polycarbonate filter with 0.67µm pores in the surface was clearly visible (Figure 4.7.a), whilst the exposed counterpart illustrated the suitability of the flat filter for distinct identification of PM_{10}, such as soot spherulites using FESEM (Figure 4.7.b). The PUF honeycomb microstructure was easily identified (Figure 4.7.c); note the heavy encrustation of PM_{10} in a 'trench' following exposure, and the areas of unsaturated PUF selected for IA (Figure 4.7.d).

4.3.3.1 LANDFILL PM_{10} COLLECTION 2007

The Negretti sampler was used to collect PM_{10} onto polycarbonate filters for

morphological and size analyses in 2007. The differences in PM_{10} density and type were visibly discernible from the filters at increasing magnification (Figure 4.8).

The two main particle types seen at the landfill were determined to be mineral and soot aggregates (Figure 4.9). Most mineral matter was present as heterogeneous composites, with a small number of salts, such as sodium chloride and gypsum, also contributing to this group (Figure 4.9.f,h). Very few examples of biological matter (e.g. pollen grains and fungal spores) were found (Figure 4.9.a, d).

4.3.3.2 URBAN PM_{10} COLLECTION 2007

The main particle types seen in the urban 2007 samples were soot aggregates and a small number of minerals (Figure 4.10). The majority of soot was present as very fine and light chains of individual, smooth and regular spherulites (Figure 4.10.a); however, some soot was also present as denser aggregate clumps (Figure 4.10.e).

The particle frequencies of landfill and urban 2007 collections revealed no significant differences between the two locations (Figure 4.11). A total of 8595 and 4944 particles were measured by IA for landfill and urban 2007 samples, respectively. Less than 1% (110 particles) of landfill PM_{10} were larger than 2.5μm, and this proportion was halved in the urban PM_{10} collection (0.5%; 31 particles).

Although FESEM-IA determined there was no significant difference between the size distribution at the two sites (Figure 4.11.a), there was a striking difference in particle composition of the two locations (Figure 4.11.b). The over-riding particle type in landfill 2007 PM_{10} was mineral (98% by numbers), whereas the dominant species at the urban site was soot (93% by numbers). Although a trace number of pollen and spore grains were detected, this amounted to less than 10 particles in the landfill and 5 particles in the urban collections, and therefore did not significantly contribute to the overall particle classification.

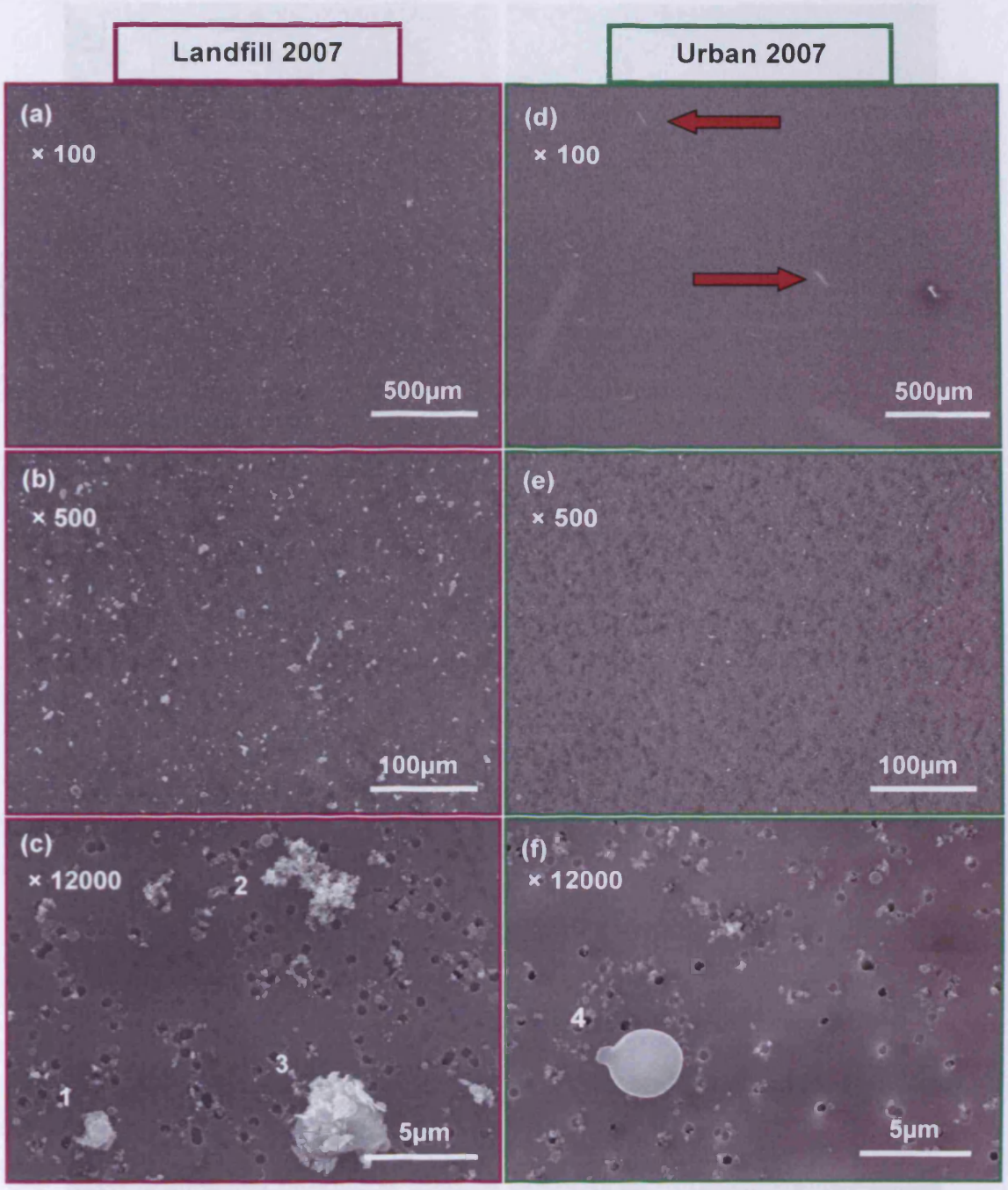

Figure 4.8: *FESEM images of Negretti PM_{10} polycarbonate filters collected at landfill and urban locations in 2007. Increasing magnification reveals the greater particle density and presence of coarse particles at the* ***(a-c)*** *landfill site, compared to* ***(d-f)*** *urban filter sections;* ***(d)*** *Filter clip holder markings (arrows) were discernible in this urban sample, due to the sparse distribution of particulate matter upon the flat surface;* ***(c,f)*** *PM_{10} types 1 = fine mineral; 2 = coarse soot aggregate; 3 = coarse mineral/soot composite; 4 = organic aeroallergen sphere.*

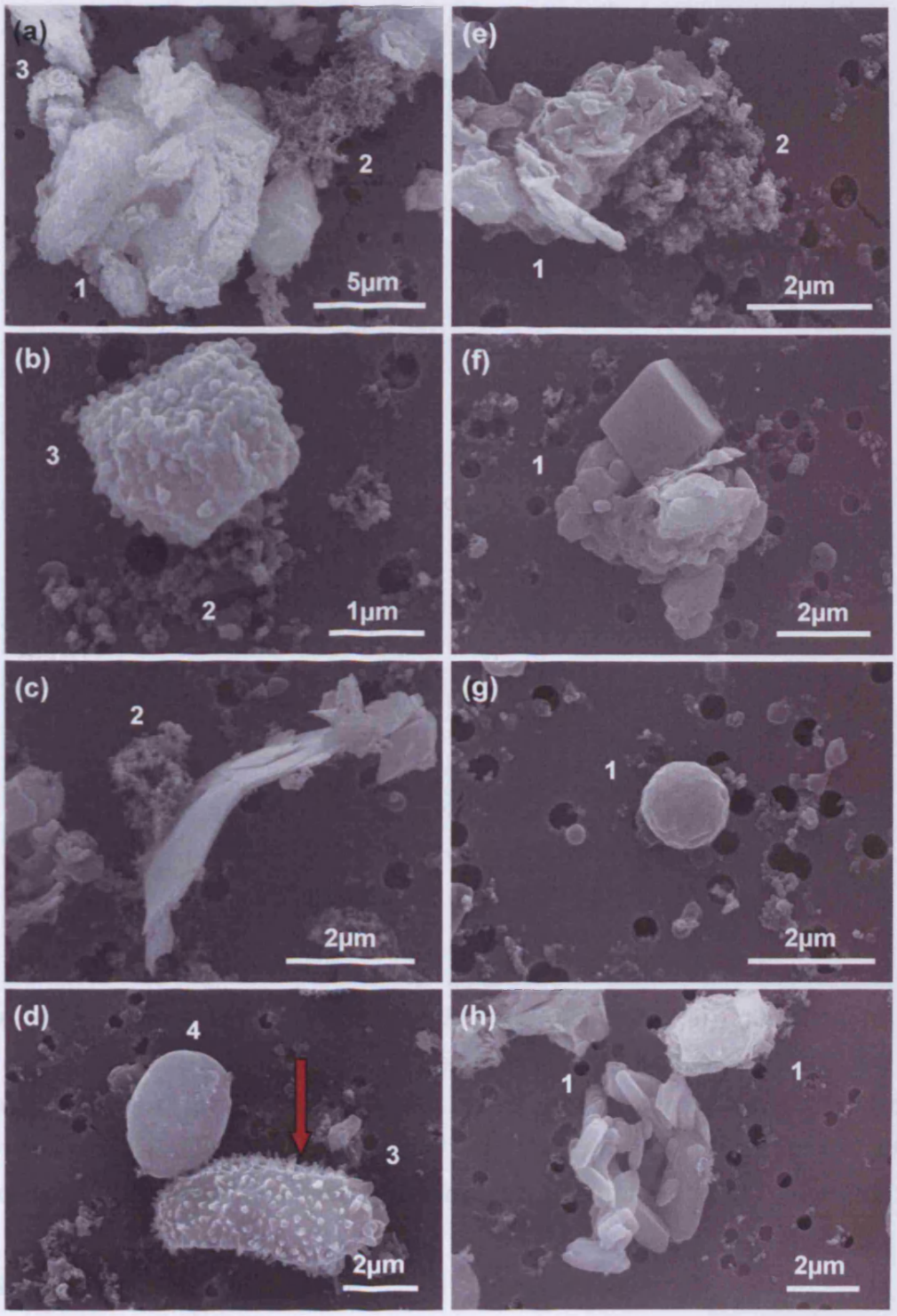

Figure 4.9: *Different particle types seen in the landfill 2007 PM_{10} collection.* ***(a)*** *large mineral aggregate particle in association with soot and pollen;* ***(b)*** *soot and pollen;* ***(c)*** *organic flake in association with minerals and soot chains;* ***(d)*** *oblong pollen (arrow) and organic particle;* ***(e)*** *soot clump with larger unidentified particle, possibly mineral;* ***(f)*** *cubic salt with mineral matter;* ***(g)*** *aluminosilicate sphere (glass);* ***(h)*** *regular gypsum crystals. Particle types 1 = mineral; 2 = soot; 3 = biological; 4 = organic particle.*

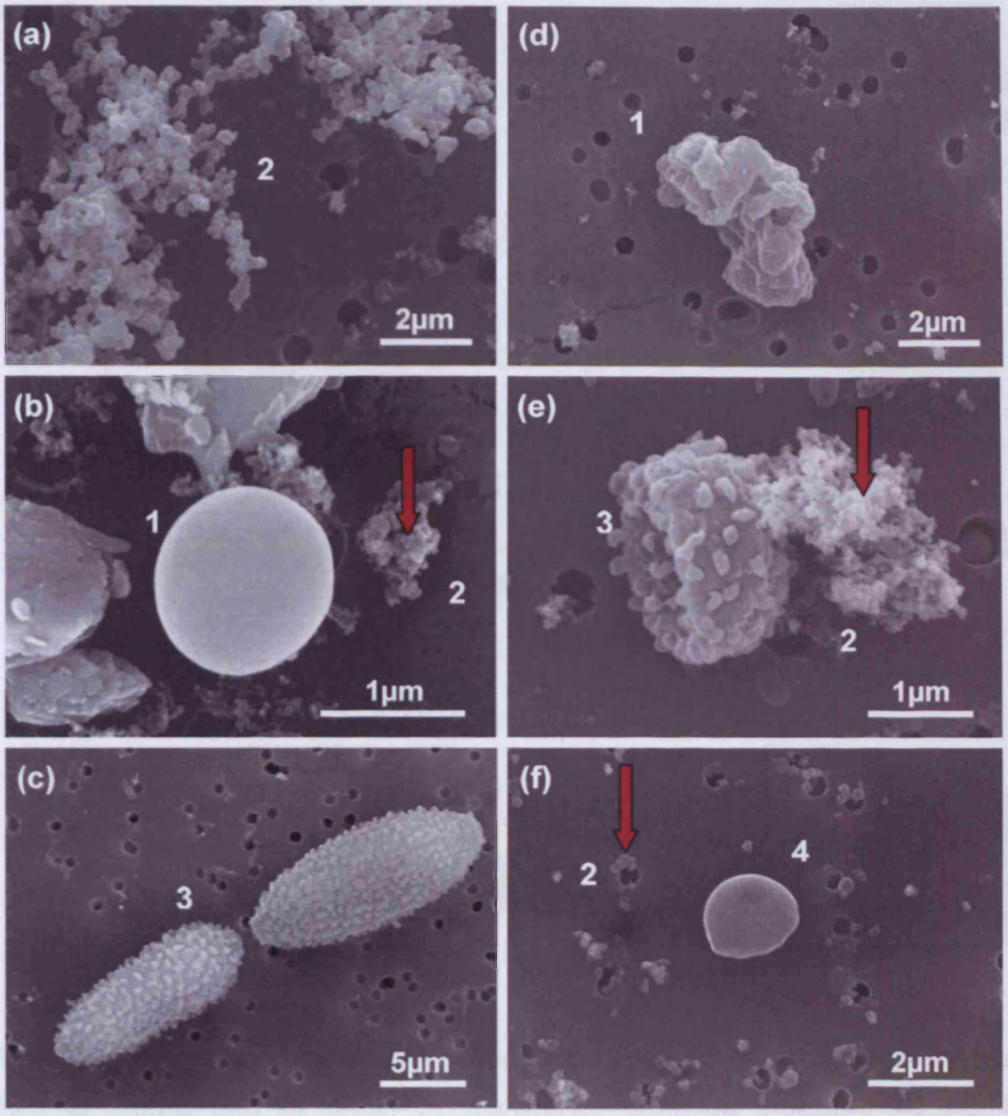

Figure 4.10: *Different particle types seen in the urban 2007 PM_{10} collection.* ***(a)*** *light soot chains;* ***(b)*** *spherical smelter particle (iron oxide) surrounded by mineral and soot (arrow);* ***(c)*** *pollen;* ***(d)*** *mineral;* ***(e)*** *soot clump (arrow) associated with pollen;* ***(f)*** *organic particle, accumulation of individual soot particles around pore edges (arrow). Particle types 1 = mineral; 2 = soot; 3 = biological; 4 = organic particle.*

4.3.3.3 PM_{10} Collections 2008

A minimum of 40 images per site were analysed in 2008, encompassing the $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ fractions. A total of 2596 (landfill) and 8981 (urban) $PM_{2.5\text{-}0.1}$ stage particles along with 970 (landfill) and 1315 (urban) $PM_{10\text{-}2.5}$ stage particles were counted in 2008. The different morphologies seen in the landfill $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ samples along with their corresponding urban fractions are illustrated in Figures 4.12 and 4.13.

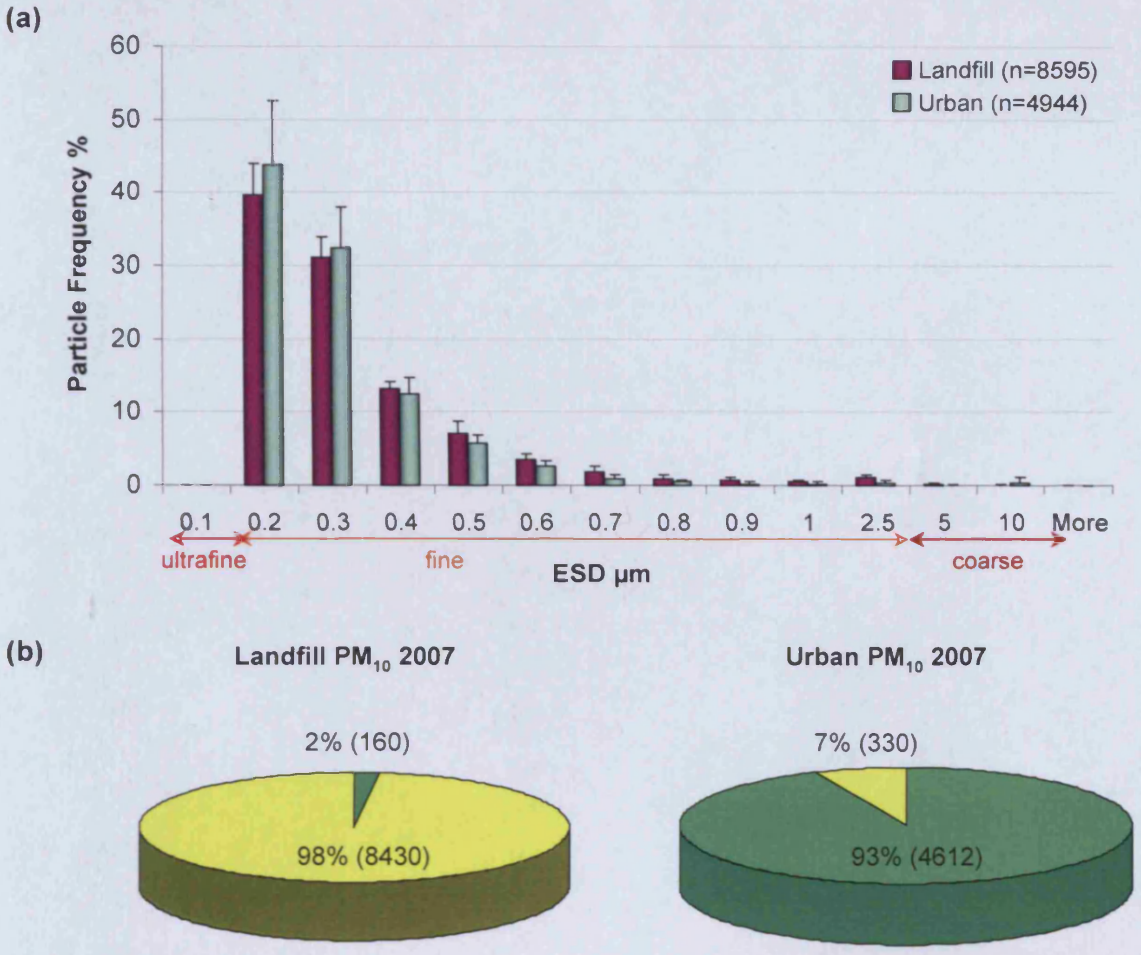

Figure 4.11: *FESEM analysis of PM_{10} collections in 2007.* ***(a)*** *Particle size distributions of PM_{10} landfill and urban polycarbonate samples indicated no significant difference between the two locations (mean ± stdev);* ***(b)*** *The composition of landfill and urban collections were dominated by crustal minerals and soot. Parentheses indicate PM_{10} number count. There was a preferential contribution of mineral to landfill PM_{10} and soot to urban PM_{10}.*

The PM_{10} collected by the HVCI were present as complexes of separate components. This was discernible in both coarse and fine landfill PUF stages. Minerals were often found in heterogeneous composite aggregates, forming larger particles (Figure 4.12.a, c, d, e). The two dominant particle types in landfill $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ were plate-like or irregular minerals (Figure 4.12.a-c) and soot (Figure 4.12.e-g), with very few biological particles identified in either stage (Figure 4.12.f). The soot was often present as aggregates in compact association with other particles, with little evidence of lighter chains.

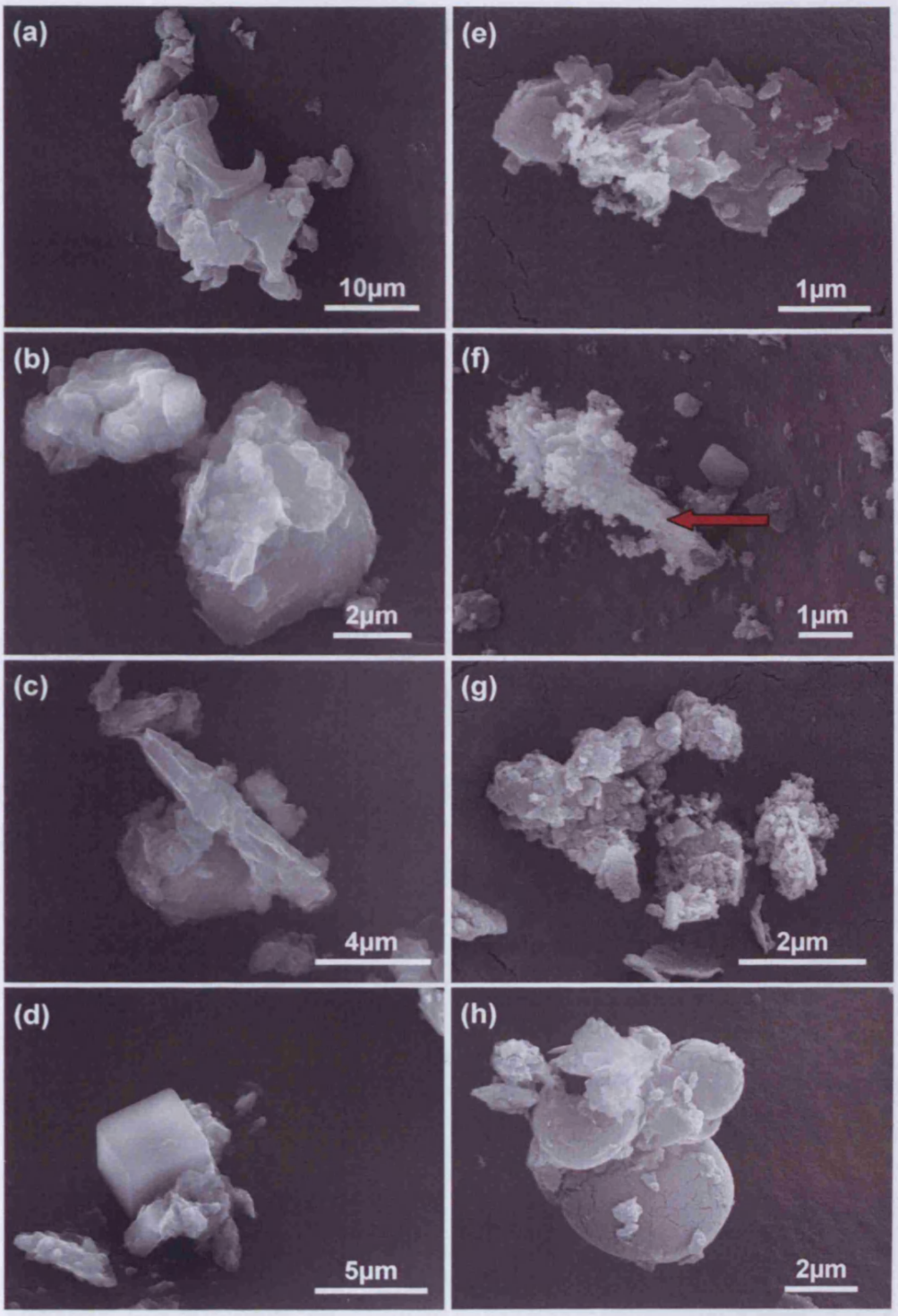

Figure 4.12: *Different types of particulate matter detected in landfill 2008* ***(a – d)*** *$PM_{10\text{-}2.5}$ and* ***(e – h)*** *$PM_{2.5\text{-}0.1}$ PUF collections. The majority of particulates were* ***(a – h)*** *heterogeneous mineral composites. Compact soot clumps were also detected in association with* ***(e, g)*** *mineral particles or* ***(f)*** *biological fragments (arrow). Relatively few* ***(d)*** *cubic salts or* ***(h)*** *fly ash particles were observed.*

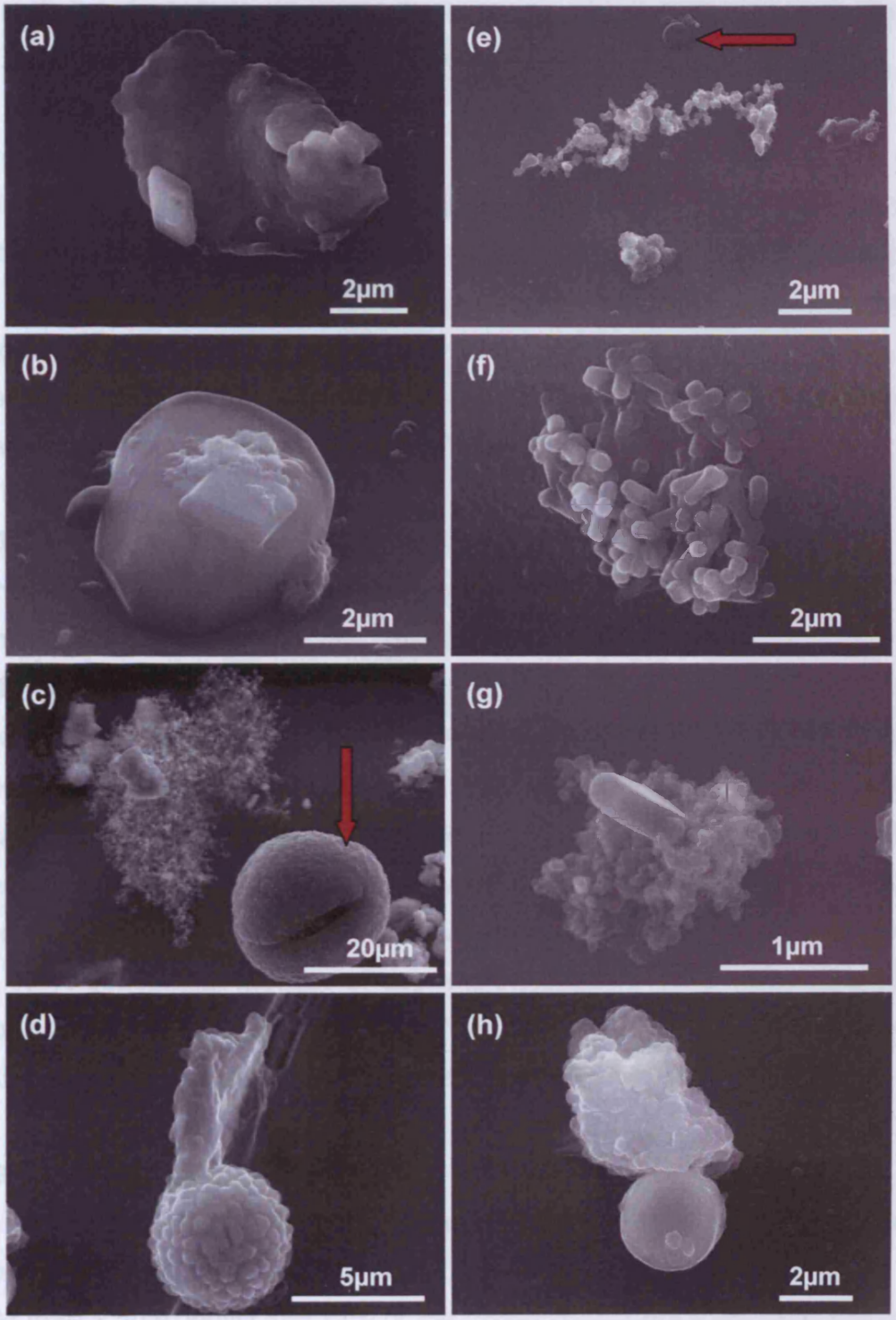

Figure 4.13: *Different types of particulate matter detected in urban 2008* ***(a – d)*** *$PM_{10\text{-}2.5}$ and* ***(e – h)*** *$PM_{2.5\text{-}0.1}$ PUF collections.* ***(a,b)*** *Salt particles seen in surface-associations with larger mineral matter;* ***(c)*** *Large fluffy soot aggregates adhere to smaller minerals and large pollen grain (arrow);* ***(d)*** *Spherical pollen sphere with organic fleck;* ***(e)*** *Soot (upper), brochosomes (lower), arrow indicates sulphur-rich droplet;* ***(f)*** *Recrystallised salt crystals;* ***(g)*** *elongated salt crystal embedded in soot aggregate;* ***(h)*** *fine iron oxide sphere in conjunction with mineral matter.*

The majority of minerals were irregular in shape, rather than cubic salts (Figure 4.12.d) or hollow fly ash particles (Figure 4.12.h).

There was more evidence of "wetting" in urban $PM_{10-2.5}$ and $PM_{2.5-0.1}$ PUF sections versus the landfill PUF samples. The urban 2008 PUF samples exhibited more over-sized mineral features (e.g. larger than 20μm), and soot, in both the coarse and fine fractions when compared to the equivalent landfill samples (Figure 4.13.b). A number of recrystallised features were also observed (Figure 4.13.f). Condensation of sulphur-rich droplets was observed in association with small soot clusters (Figure 4.13.e).

4.3.3.3.1 $PM_{2.5-0.1}$ Collections 2008

Neither landfill nor urban fine fraction samples contained particles greater than 10μm, whilst just 14% and 4% (respectively), were larger than 2.5μm. The landfill and urban 2008 $PM_{2.5-0.1}$ size distributions were not statistically different to each other. However, the landfill sample displayed a significantly greater proportion of particulates larger than 1μm compared to the urban sample (Figure 4.14.a; $p \leq 0.05$). Fewer landfill fine particles were detected (approximately 70% less) when compared to the number of urban $PM_{2.5-0.1}$ particles. The majority of the fine PM were either soot or mineral (Figure 4.14.b). A substantial number of sub-micron droplets were also observed on various urban PUF $PM_{2.5-0.1}$ sections. The average ESD of these droplets was 0.7μm, and these were absent from landfill samples.

4.3.3.3.2 $PM_{10-2.5}$ Collections 2008

The landfill and urban 2008 $PM_{10-2.5}$ distributions were not statistically different to each other (Figure 4.15), although 27% of the landfill coarse fraction was larger than 2.5μm, whilst just 12% of the urban coarse particles were above this size. Similar particle numbers and proportions of landfill and urban 2008 coarse mineral and soot particles were obtained (Figure 4.15.b). Although not to the same extent as the fine fraction, a 25% decrease in the number of landfill $PM_{10-2.5}$ particles compared to the urban coarse collections was observed.

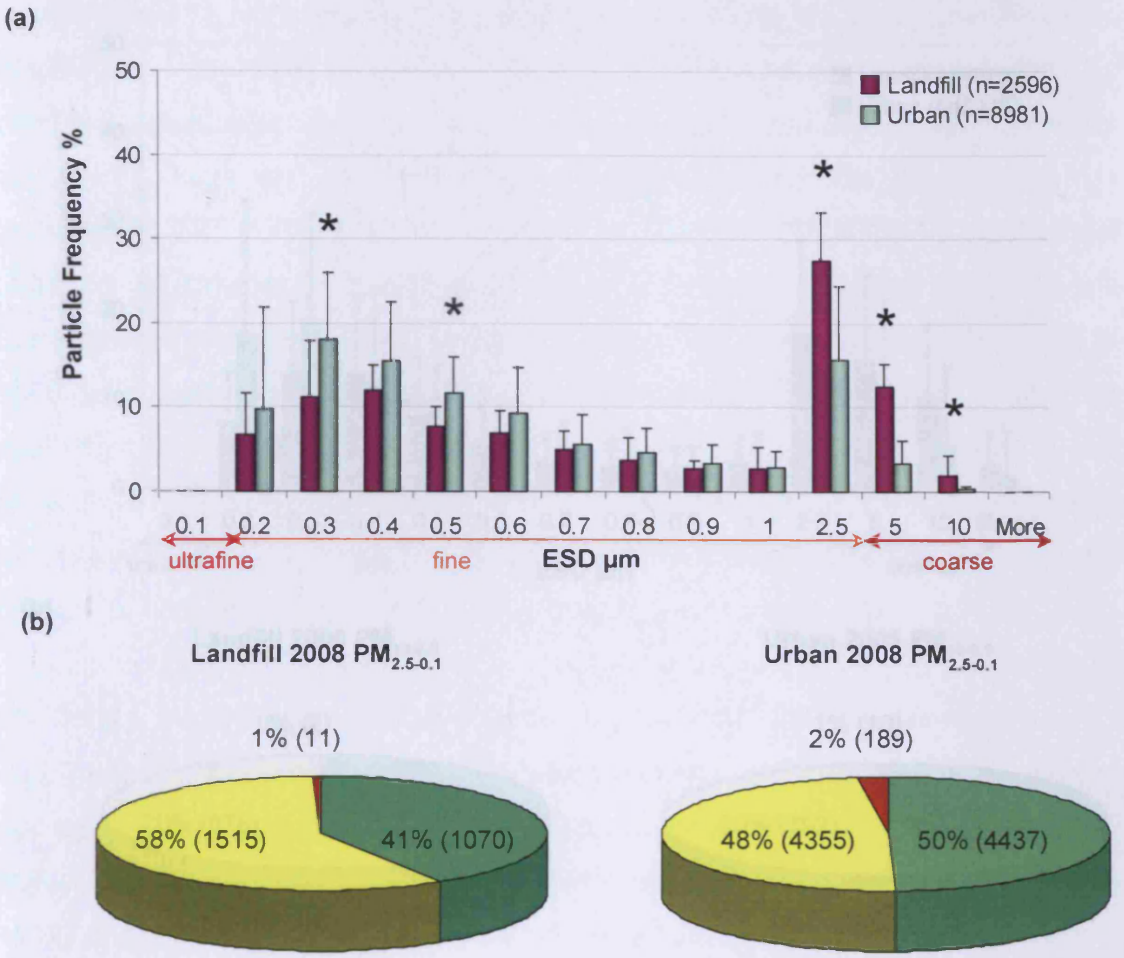

Figure 4.14: *FESEM analysis of landfill and urban $PM_{2.5-0.1}$ collections in 2008.* ***(a)*** *Particle size distributions of $PM_{2.5-0.1}$ ■ landfill and ■ urban PUF samples indicate similar size trends, but a much larger proportion of landfill particles at the upper range of the fine fraction (mean ± stdev).* ***(b)*** *The composition of landfill and urban collections were dominated by ■ minerals and ■ soot, with less than 5% of numbers attributable to ■ biological components in either sample. Parentheses indicate PM number count, revealing the largest number of particles was collected by the urban $PM_{2.5-0.1}$ stage. * represents $p \leq 0.05$ inter-site. The peak seen at 2.5-10µm is an artefact of the increased histogram interval.*

4.4 DISCUSSION

The primary aim of this investigation was to evaluate the physical characteristics of landfill airborne PM_{10} in comparison with urban PM_{10}. To fulfil this, two successive summer seasons (2007 and 2008) were monitored at a

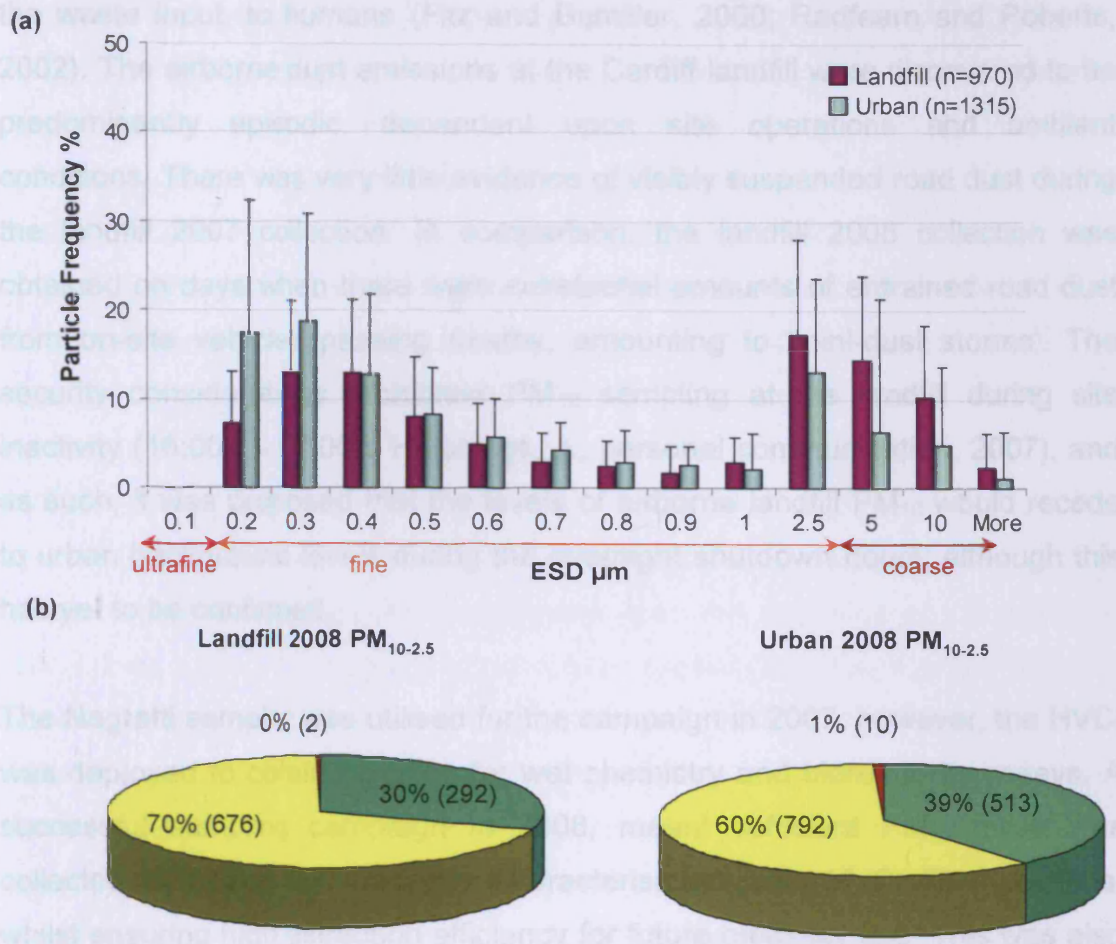

Figure 4.15: *FESEM analysis of landfill and urban $PM_{10-2.5}$ collections in 2008.* ***(a)*** *Particle size distributions of $PM_{10-2.5}$ ■ landfill and ■ urban PUF samples indicate fewer landfill than urban coarse particles were obtained, yet both displayed similar size trends (mean ± stdev).* ***(b)*** *The composition of landfill and urban collections were dominated by ■ minerals and ■ soot, with a negligible proportion of ■ biological components in either sample. Parentheses indicate PM number count, and reveal the smaller number of particles collected by the landfill $PM_{10-2.5}$ stage. The peak seen at 2.5-10µm is an artefact of the increased histogram interval.*

Cardiff municipal landfill (landfill A; Lamby Way waste site), with accompanying sampling performed at an inner-city urban location (Cardiff University). In addition to gravimetric mass concentrations, FESEM-IA was used for identification of particle morphology and numbers for each collection.

Anthropogenic activity upon a landfill gives rise to airborne particulate matter, which may provide an exposure pathway of a wide variety of chemicals from

the waste input, to humans (Fitz and Bumiller, 2000; Redfearn and Roberts, 2002). The airborne dust emissions at the Cardiff landfill were discovered to be predominantly episodic, dependent upon site operations and ambient conditions. There was very little evidence of visibly suspended road dust during the landfill 2007 collection. In comparison, the landfill 2008 collection was obtained on days when there were substantial amounts of entrained road dust from on-site vehicles passing nearby, amounting to 'mini-dust storms'. The security considerations prohibited PM_{10} sampling at the landfill during site inactivity (16:00h – 07:00h; Hutchings, J., personal communication, 2007), and as such, it was proposed that the levels of airborne landfill PM_{10} would recede to urban background levels during the overnight shutdown hours; although this has yet to be confirmed.

The Negretti sampler was utilised for the campaign in 2007, however, the HVCI was deployed to obtain samples for wet chemistry and bioreactivity assays. A successful sampling campaign in 2008, meant sufficient PM_{10} mass was collected to permit simultaneous characterisation from HVCI filter sections, whilst ensuring high extraction efficiency for future bioassay use. This was also considered to be a method improvement, as many researchers highlight the importance of obtaining physicochemical speciation and bioreactivity response from a universal PM_{10} population (Pennanen *et al.*, 2007). The morphological characteristics of the two seasons of collections could be compared to a certain extent. However, as the two data sets were derived from different devices (Negretti sampler collection PM_{10} in 2007 and HVCI sampler collecting $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ in 2008), these comparisons have been made cautiously. The HVCI System I was used for early collections, however, the logistical demands of sampling on an operational landfill waste mass resulted in migration to System II, which was trailer-mounted, and easier to transport. Previous investigators have shown that the two different flow rates of 900L/min and 1100L/min did not significantly affect the cut-off size distributions in the three HVCI stages (Moreno *et al.*, 2003, 2004b; Jones *et al.*, 2006).

Sampling at the MSW site was restricted to summer seasons due to meteorological and logistical factors. The 2008 landfill sampling campaign was

the only occasion when PM_{10} levels exceeded current UK National Air Quality guidelines of 50µg/m^3. However, as this Government objective was a 24h mean value, the mass concentrations calculated from the various sampling campaigns were not directly comparable as it was not possible to monitor continuous 24h periods at the landfill due to site inaccessibility. This also meant that although vital to site characterisation, the extrapolation of gravimetric mass concentration measurements, to the long-term air quality indicators, was not an appropriate use of this data (Macleod *et al.*, 2006).

FESEM-IA can enable the classification of the major particle types within a given PM population; nonetheless, it was subject to user-error, as correct categorisation of each particle was based upon the expertise of the operator, rather than an individual particle's chemical profile. The composition of MSW was inevitably diverse, yet household waste constituted a significant part of the daily disposal into municipal landfills. Hence, the low numbers of biological particles (e.g. pollen grains; fungal spores) in both campaigns was an unexpected finding, due to the variable organic, biodegradable nature of domestic waste. With the EU Landfill Directive legislating towards waste minimisation (European Council Directive 1999/31/EC), the Lamby Way landfill site has been operating a large-scale composting facility, accommodating kerbside collections since 2006 (Hutchings, S., personal communication, 2009). As the PM_{10} collectors were located approximately 50m above and 750m away from these compost heaps, it was plausible that the landfill PM_{10} of both campaigns was influenced to a greater extent, by vehicular emissions and the most recent waste deposits, containing less biodegradable materials.

4.4.1 PM_{10} Collections 2007

FESEM-IA of landfill and urban PM_{10} revealed a similar size distribution at these two locations; however, it also revealed significantly increased PM_{10} generation at the landfill compared to the urban location, i.e. 42 and 13µg/m^3, respectively. The physical distribution of PM_{10} upon the filters was much more concentrated in the landfill 2007 collection, and this was reinforced by the total number of particles detected by IA, being nearly double that of the urban collection.

Although PM_{10} smaller than 0.1µm (i.e ultrafines), such as soot particles in and around filter pores, were visible during inspection of electron micrographs, this was below the detection limit of the QUIPS software IA routines, and therefore, unquantifiable. Nonetheless, this may have lead to preferential detection of these smallest sized PM_{10} during IA and both collections exhibited a skewed ESD distribution, with over 99% of all PM_{10} appearing to be less than 1µm ESD. It must be noted that as the bin ranges change in order to display the complete size dataset on a single axis, the increases in particle frequency towards the upper end of the histogram scale represented an artefact.

The landfill and urban 2007 samples exhibited the same ESD distribution. Anthropogenic activities at the operational landfill would have been expected to suspend crustal dust, which might then have been expected to fall into the coarse fraction. Thus, the lack of size difference between the Negretti (2007) landfill and urban samples was counter-intuitive, when considering the dissimilarity in IA particle classification, or the mass concentrations according to the HVCI collection stages. Unfortunately, no PUF sections were reserved for morphological analysis from the 2007 campaign, due to the large masses of sample required for the bioassays. Limited information about particulate numbers can be garnered from mass measurements, but the higher landfill $PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ mass concentration ratio (HVCI collection), the absence of dense smelter particulates and the significant crustal mineral component of landfill PM_{10} (Negretti collection), all support the finding of a greater particle number count at the landfill compared to the urban site.

The morphology of the landfill and urban PM_{10} were strikingly different. The main particle types were identified as either mineral or soot, with 98% of landfill PM numbers attributed to predominantly irregular-shaped mineral particulates, whilst soot accounted for 93% of the urban PM_{10}. The soot was present as aggregates of smooth, uniform 20-30nm spherulites, indicating high temperature combustion processes (BéruBé *et al.*, 1999). It was interesting to note the lack of significant (cubic) sea salt in the landfill PM_{10} coarse mode mineral population. This was unexpected, due to the proximity of the landfill to the Severn Estuary, a source of wind-blown sea salt (Godoy *et al.*, 2009). Both

sites generated PM_{10} with the vast majority of particles distributed within 2.5-0.1μm aerodynamic diameter, and therefore, highly respirable (Churg and Brauer, 2000; WHO, 2003).

4.4.2 PM_{10} COLLECTIONS 2008

Gravimetric analysis revealed an extremely high landfill PM_{10} mass concentration of 112μg/m^3, over twice the recommended national guidelines, whilst the Cardiff urban PM_{10} mass concentration of 5μg/m^3 was half the value obtained in a previous study (10μg/m^3 in Moreno *et al.*, 2004b). The landfill 2008 $PM_{2.5\text{-}0.1}$:$PM_{10\text{-}2.5}$ mass concentration ratio revealed a much weaker traffic-related signature at the landfill site, compared to either the accompanying urban, or previous landfill 2007 collections. This also supported the on-site observation of significant soil suspension episodes during 2008 landfill sampling. Morphological characterisation of landfill and urban PM_{10} as $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ in 2008 was possible from HVCI samples, based upon the premise that the particles found at the fringe of the collection trench, were good representations of the total PUF sample. This methodology has been confirmed by previous investigations of the HVCI (Moreno *et al.*, 2003, 2004b; Jones *et al.*, 2006). The separation of fine and coarse modes by the HVCI enabled more specific characterisation of the PM_{10} population compared to the Negretti collections of 2007.

The 2008 size analyses of both $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ were obtained from a larger number of electron micrographs than in 2007 (Negretti filters). The FESEM-IA of PUF sections was complicated by the undulating nature of the collection substrate. Although maximal use of peripheral PUF regions provided adequate particles for sizing and composition data, fewer particles were detected for both locations when compared to the 2007 collection. This was a striking discovery, in view of the much larger masses collected at both sites in 2008, and indicated a collector-dependent effect upon PM_{10} characterisation. The mechanism of impaction and filtration of the PUF substrate inferred that in practice, a significant proportion of PM_{10} became trapped within the 2-3mm deep trench formed below the impaction slits, with the edges becoming

populated by disperse semi-filtered and bounce-back particulates (Jones *et al.*, 2007). Since the vast majority of particulates were sized at ≤2.5μm in the $PM_{2.5-0.1}$ stage and at ≤10 μm in the $PM_{10-2.5}$ stage, this confirmed the HVCI was operating as intended.

Regardless of sampling location, all 2008 $PM_{2.5-0.1}$ and $PM_{10-2.5}$ fractions exhibited bimodal size distributions, with fine and coarse particles observed in both collection stages of the HVCI. This was a pertinent finding in the $PM_{10-2.5}$ stage, with possible causes being (i) the filtering effect of the PUF substrate, causing deposition of PM_{10} at trench edges and (ii) the weak-associations between coarse PM_{10} aggregates, causing composite particles to fracture into their component parts upon impaction (Sexton, K., personal communication, 2009).

The morphology of landfill 2008 PM_{10} showed similar trends to urban 2008 PM_{10}, for both $PM_{10-2.5}$ and $PM_{2.5-0.1}$ modes. The urban 2008 $PM_{2.5-0.1}$ sample exhibited considerably less soot than in the urban PM_{10} 2007 (Negretti) sample. When considering the possible retardation of soot chains and clusters by larger, immobile PM_{10} within the central PUF trench, the finding of just 50% soot in $PM_{2.5-0.1}$ urban 2008 (compared to 93% soot observed in the urban PM_{10} 2007 sample), was not altogether surprising. For both landfill and urban samples, there was an approximately 10% decrease in the number of soot particles in the $PM_{10-2.5}$ fraction compared with the corresponding $PM_{2.5-0.1}$ - mainly to the benefit of mineral numbers. There were a significant number of highly respirable (0.7μm) sulphur-rich droplets observed in the urban 2008 fine fraction, and these were postulated to be due to asphalt paving (road building) activities occurring approximately 50m from the urban collection site, or from unburnt diesel-engine emissions (Lighty *et al.*, 2000). Several over-sized cubic mineral PM were observed in the urban 2008 PUF samples, yet these were absent from the landfill 2008 samples. They were most likely formed from recrystallisation of soluble salts, aided by possible moisture ingress into the HVCI collection head during the extended sampling regime at the urban site. This finding may indicate a relatively insoluble mineral composition at the

landfill site, and a greater soluble (salt) component within the urban mineral population (Chapter 5).

The largest number of particles in 2008 was detected in the urban $PM_{2.5-0.1}$ sample, whereas there were dramatically fewer landfill $PM_{2.5-0.1}$ particles. This relationship was also noticed in the coarse fraction, meaning both landfill $PM_{2.5-0.1}$ and $PM_{10-2.5}$ samples had a smaller surface area than the corresponding urban 2008 samples. As increased PM surface area has often been linked to rises in toxicity (Brown *et al.*, 2001; Donaldson *et al.*, 2002; Valavanidis *et al.*, 2008), this decrease in surface area of the landfill 2008 collection may indicate a decreased bioreactivity in comparison to both the corresponding urban 2008 and the previous landfill (2007) PM_{10} (Chapter 6).

4.5 CONCLUSIONS

In summary, elevated mass concentrations revealed larger masses of airborne particulates could be generated at the landfill, yet when compared to the parallel urban samples, particle numbers appeared to vary, depending on the collection device and sampling occasion.

Lamby Way landfill airborne particulates in 2007 and 2008 were predominantly crustal minerals, with fewer sea salts than complementary urban PM_{10} samples, which were dominated by soot particulates. Although a substantial percentage of soot particles were also present in the landfill 2008 fine fraction, this translated to a smaller number of particles when compared to the corresponding urban collection in either 2007 or 2008. The absence of biogenic particles in landfill PM_{10} was surprising, considering the variable domestic nature of municipal solid waste and the affiliation of the landfill site with a nearby composting facility.

All HVCI samples reported in this chapter were extracted from PUFs and processed for inorganic chemical composition (Chapter 5), before proceeding with *in vitro* toxicity testing (Chapter 6).

CHAPTER 5

CHEMICAL CHARACTERISATION OF PARTICULATE MATTER

5.1 INTRODUCTION

A number of epidemiological studies have shown an association between long-term exposure to ambient PM_{10} and morbidity and mortality (WHO, 2003; Pope *et al.*, 2004); yet there are uncertainties regarding the mechanisms by which inhaled particulates exert their toxic effects. Several physicochemical factors have been highlighted as protagonists - particle size, mass concentration, morphology (Chapter 4), structure and chemical reactivity (Harrison and Yin, 2000) – however, it is likely that different particle properties trigger different cellular endpoints.

The chemical composition of PM_{10} is highly heterogeneous and variable, resulting from the varied sources and dispersal processes which form airborne particulates. PM_{10} comprises of primary and secondary particles. Primary particles are formed directly from emission sources, including crustal (geological, sea-spray) and indirect anthropogenic (combustion condensation, road salting) activity. In contrast, secondary particles are a result of gas-to-particle conversions and incorporate sulphates, nitrates and volatile organic species; these are formed from photochemical atmospheric processes; often resulting in more soluble airborne pollutants (EPAQS, 2001).

There is a growing body of evidence supporting the role of biochemically reactive transition metals in the hazard that PM_{10}, and in particular, the smaller size fractions, $PM_{2.5}$ and $PM_{0.1}$, pose to human health (Donaldson *et al.*, 2002; Schaumann *et al.*, 2004; Sørenson *et al.*, 2005; Valavanidis *et al.*, 2008). To focus the research, it was decided to limit the experimental work to the inorganic fraction of the PM_{10}, given this has already been highlighted as a probable mediator of the observed PM_{10}-mediated bioreactivity and health effects (Schaumann *et al.*, 2004; Ghio *et al.*, 2005; McNeilly *et al.*, 2005; Sørensen *et al.*, 2005).

The aims of this section of the research were to:

- Investigate the *in situ* bulk chemical composition of landfill PM_{10} (2007),

$PM_{2.5-0.1}$ and $PM_{10-2.5}$ fractions (2008) by FESEM-EDX

- Elucidate the major and trace elemental metal profile of bulk and aqueous fractions of landfill and urban $PM_{2.5-0.1}$ and $PM_{10-2.5}$ by ICP-MS
- Quantify the concentrations of sulphate, chloride and nitrate anions in aqueous extracts of the landfill and urban $PM_{2.5-0.1}$ and $PM_{10-2.5}$ by IC.

5.2 METHODS

5.2.1 MATERIALS AND SOURCES All sources are UK-based unless otherwise stated

Materials and Equipment:	Source:
18 MΩ Water	In-house, Cardiff University
A&D GR202 Analytical Balance	A&D Weighing, CA, USA
Anion Analysis Chromleon® IA Software	Dionex
Anion Analytical Column IonPac® AS15	Dionex
Anion Dionex 1200 IC System	Dionex
Anion Self Regenerating Suppressor Ultra II	Dionex
Automated Sampler AS	Dionex
Carbon Black CB M120	Cabot Corp., MA, USA
Conductivity Detector Cell DS6	Dionex
Eppendorfs (NoStick™; Sterile)	Alphalabs
Falcon tubes (Polypropylene)	Greiner Bio-One
INCA Energy X-ray Analysis System(EDX)	Oxford Instruments
K450 Sputter Coater (Carbon)	Emitech
Millex® Syringe Filters (MCE,0.22µm)	Fisher Scientific
MDS-2000 Microwave	CEM Corp. NC, USA
Nitric Acid, HNO_3 Analytical Grade, 69%	Fisher Scientific
Pirani 10 Lyophiliser	Edwards
Philips XL30-FEG SEM (FESEM)	Philips Electron Optics, Eindhoven, NL
Rupture Membranes	CEM Corp., NC, USA
Thermo X7 ICP-MS	Thermo Electron Corporation,
Ultrasonicator Bath, 50Hz	Kerry Ultrasonics
Vortex Genie 2	Jencons

Whatman Puradisc Syringe Filters (CA, 0.2µm)	GE Healthcare Life Sciences

5.2.2 HVCI Substrate Extraction

The HVCI substrates (PUFs) were cut into 6 equal sections before placing each section into pre-weighed 15ml Falcon polypropylene tubes; in the case of $PM_{2.5-0.1}$ filters, 2 sections were combined per extraction tube. The extraction of PM from the PUF filters was carried out at 80% recovery (Table 5.1).

Extraction scheme	Duration
Falcon tubes weighed (to 4 d.p)	
7ml 18 MΩ water added to pre-weighed tube, immersing PUF section (Extract #1)	
Horizontal agitation (vortex setting 7, medium/fast)	2h
Sonication	2min
PUF section transferred to fresh Falcon tube; Extract #1 stored at -20°C	
7ml 18 MΩ water added to each tube, immersing each PUF section (Extract #2)	
Horizontal agitation (vortex setting 7, medium/fast)	2h
Sonication	2min
PUF section compressed with sterile syringe, maximising Extract #2	
Extract #2 combined with Extract #1	
$PM_{2.5-0.1}$ or $PM_{10-2.5}$ suspension freeze-dried	~20h
Falcon tubes re-weighed (to 4 d.p)	
Freeze-dried PM mass obtained	
Stock solution (2-10mg/ml) in molecular biology H_2O stored at -80°C	

Table 5.1: *PUF extraction protocol for isolation of $PM_{2.5-0.1}$ and $PM_{10-2.5}$ for wet chemical composition and bioreactivity assays (Jones et al., 2006). Buffer extraction was avoided to minimise sample-matrix interferences in either chemical or bioreactivity analyses.*

5.2.3 *FESEM – EDX*

Negretti filters were used for FESEM energy-dispersive X-ray analysis (FESEM-EDX) in 2007 but the analyses for 2008 were performed on HVCI PUF sections as a method development.

5.2.3.1 *FESEM-EDX SAMPLE PREPARATION*

Filter samples were prepared as described in Section 4.2.7, with the exception that the stubs were carbon coated from a graphite source (K450 Sputter Coater). Duplicate stubs were prepared for each Negretti filter collected. Triplicate stubs of PUF samples obtained in 2008 were also prepared.

Individual particle chemical analyses were performed using EDX linked to a Philips XL30 FEG environmental scanning electron microscope (FESEM) in Back Scatter Electron (BSE) mode. Spectra were obtained from the centre of particles at an accelerating voltage of 20kV, 5000x magnification, working distance 10mm, beam current 1nA, spot size 4, and an acquisition time of 30s. A minimum of 9 areas of each filter section (minimum 300 analyses per site sampled) were analysed. The data were stoichiometrically adjusted for oxygen to account for natural oxidation processes, and exported as weight percentage (weight %) into MS Excel 2003 for further analysis. Major elements were identified as silicon, aluminium, sulphur, chloride, calcium and iron. In each case, a major component was identified from each EDX spectra with the following equation (Moreno *et al.*, 2003):

$$\textbf{Element of interest} \div \sum \textbf{(All elements detected)} > \textbf{5 wt\%}$$

The frequency at which each element of interest was ascertained as a major component of the spectra, for each particulate matter sample, per given site, was then calculated for comparison.

5.2.4 *ICP-MS*

The lyophilized $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ were quantitatively analysed for a full

range of metals by ICP-MS as the 'total' and 'water-soluble' portions. A closed vessel CEM MDS-200 microwave system was used to prepare total PM solutions, whilst an aqueous extraction of PM sub-samples was used to obtain the water-soluble i.e. potentially 'bioavailable' fraction of the PM.

5.2.4.1 *TOTAL PM_{10} SAMPLE PREPARATION*

Prior to use, all digestion vessels were cleaned by heated immersion in 10% HNO_3 (24h), followed by three 18MΩ water washes, and then allowed to dry (Karthikeyan *et al.*, 2006). Stock solutions were aliquoted in duplicate into pre-weighed, non-stick, 1.5ml eppendorfs and freeze-dried to provide recorded masses of 500-800µg. All weights were taken in triplicate (g), on an analytical balance (to 5 d.p). The particulate samples were transferred with 5ml 69% HNO_3 into a CEM moderate pressure vessel. The closed vessel assembly included rupture membranes at the vent hole of each cap, and vessel cuffs. In addition, care was taken to ensure the pressure control vessel with a designated cap assembly received the heaviest sample. Each prepared vessel was loaded into the carousel and placed on the microwave turntable. Vessels were subject to 80psi over a period of approximately 20min, at a digestion temperature of approximately 180°C. Samples were allowed to cool in the microwave, before manual venting. The extracts were carefully diluted to a final volume of 50ml with 18MΩ water and transferred to HDPE sample vials, yielding an acidified solution containing the total PM metal content. The samples were filtered at 0.22µm and stored at RTP until analysis (McDonald, I., personal communication, 2007).

Reproducibility and baseline element concentrations were checked with the inclusion of a procedural blank of the carbon black CB M120 as a metal-free negative control PM_{10} (Murphy *et al.*, 1998; Greenwell *et al.*, 2002), and a solvent (HNO_3) blank in each digestion cycle.

5.2.4.2 *WATER-SOLUBLE PM_{10} SAMPLE PREPARATION*

Aliquots of the stock $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ solutions prepared in Table 5.1 were diluted with 18MΩ water to yield 100µg/ml; 3ml in polyethylene flasks. The

water-soluble fraction of each PM suspension was obtained as outlined in Table 5.2.

WATER-SOLUBLE PM EXTRACT	DURATION
$PM_{10-2.5}$ or $PM_{2.5-0.1}$ suspension (100µg/ml; 3ml)	
Agitation (vortex setting 5; medium)	16h
Suspension filtered at 0.2µm	
Extracted water-soluble fraction stored at -80°C until analysis	

Table 5.2: *Water-soluble extract preparation of $PM_{2.5-0.1}$ and $PM_{10-2.5}$ for chemical composition and bioreactivity assays.*

A 1ml aliquot of the water-soluble fraction was diluted to 5ml with 10% HNO_3 and stored at RTP prior to analysis for major and trace metals. A procedural blank of 18MΩ water was processed in parallel with the soluble PM_{10} fraction.

A Thermo X7 ICP-MS was used in the metal element analyses of the total and water-soluble fractions of $PM_{10-2.5}$ and $PM_{2.5-0.1}$. The ICP-MS was operated by I. McDonald, Cardiff University; data were corrected for solvent blanks during analyses.

5.2.5 IC

Ion exchange chromatography (IC) was used to determine the concentrations of chloride (Cl^-), nitrate (NO_3^-), and sulphate (SO_4^{2-}) in the aqueous extracts of $PM_{2.5-0.1}$ and $PM_{10-2.5}$.

The ICS-1200 was calibrated at concentrations ranging between 0.1-64.5µg/l for each anion of interest. Samples (refer to Table 5.2 for preparation) were loaded into 1.5ml vials fitted with limited-volume inserts and placed in sequence into the automated sampler. All samples were analysed in triplicate. Multiple injections of 18MΩ water were analysed as controls at the start of each run to check the baseline stability, and in between every 10 injections to confirm the

absence of sample carryover. A mixed-anion standard was also run every 10 injections to evaluate peak drifting (Tang, X., personal communication, 2007).

5.2.6 *Statistical Analysis*

Data were assessed for normality with the Anderson-Darling test for homogeneity in Minitab 15 (Microsoft Inc., WA, USA). All further statistical analyses were performed in SPSS 14.0 (SPSS Inc. IL, USA). One-way ANOVA with Bonferroni corrections were used to discern any significant differences in data which were parametric. The Kruskal-Wallis test was used to investigate data which were proven to be non-parametric. *Post-hoc* testing of significant results in the Kruskal-Wallis test was done by the Mann-Whitney test. Significance was accepted at $p \leq 0.05$, whilst $p \leq 0.01$ indicated high statistical significance.

5.3 RESULTS

5.3.1 *FESEM – EDX*

All EDX analyses of 2007 utilised Negretti sections (Figure 5.1), whilst data for 2008 were obtained from PUF substrate sections (Figure 5.2).

5.3.1.1 PM_{10} *Collections 2007*

The landfill 2007 sample was dominated by Ca and Si, with a strong Al signal. There was a significantly weaker S signature in the landfill sample compared to the urban sample (p <0.01); the latter was predominantly composed of S-bearing particles. The proportion of other species contributed less than 10% of the landfill collection, but over 20% of the urban sample.

5.3.1.2 PM_{10} *Collections 2008*

The EDX results of the landfill 2008 PUF $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ data sets did not reveal a significant chemical difference between the two modes (Figure 5.2.a; One-way ANOVA, p >0.05). The geological elements Si, Ca and Al were not restricted to the coarse fraction and were also present in the fine mode.

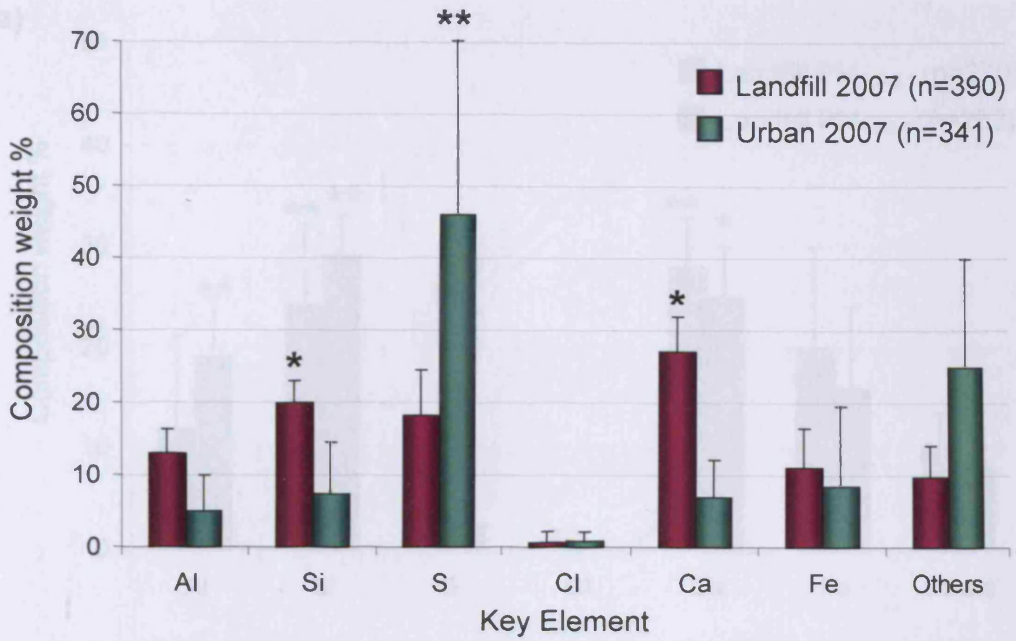

Figure 5.1: *FESEM-EDX 2007 analyses of Negretti filter collections from* ■ *landfill and* ■ *urban locations (mean ± stdev). The landfill PM_{10} collection was dominated by Ca, Si and S; the urban collection was dominated by S.* ***** *$p \leq 0.05$,* ****** *$p \leq 0.01$ inter-site significance.*

In contrast to the landfill 2008 PUF collection, the urban 2008 PUF samples exhibited significant EDX differences between the $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ modes (Figure 5.2.b; One-way ANOVA, $p \leq 0.05$). The urban $PM_{2.5\text{-}0.1}$ sample was dominated by S, to a highly significant degree when compared to the $PM_{10\text{-}2.5}$ fraction ($p \leq 0.01$). The urban $PM_{10\text{-}2.5}$ fraction exhibited a significantly greater proportion of Cl and Ca, compared to the fine mode (Figure 5.2.b). The other (trace) elements detected in varying amounts included Na, Mg, K, Zn, Mn and Cu; these contributed less to the landfill collection when compared to the corresponding urban collections.

5.3.2 ICP-MS

The total (insoluble plus water-soluble) and water-soluble metal contents of the $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ fractions are presented in airborne mass concentrations, with recommended regulatory standards for elements of toxicological significance (ng/m^3; Tables 5.3 and 5.4), and in absolute concentration (µg/g; Figures 5.3 and 5.4).

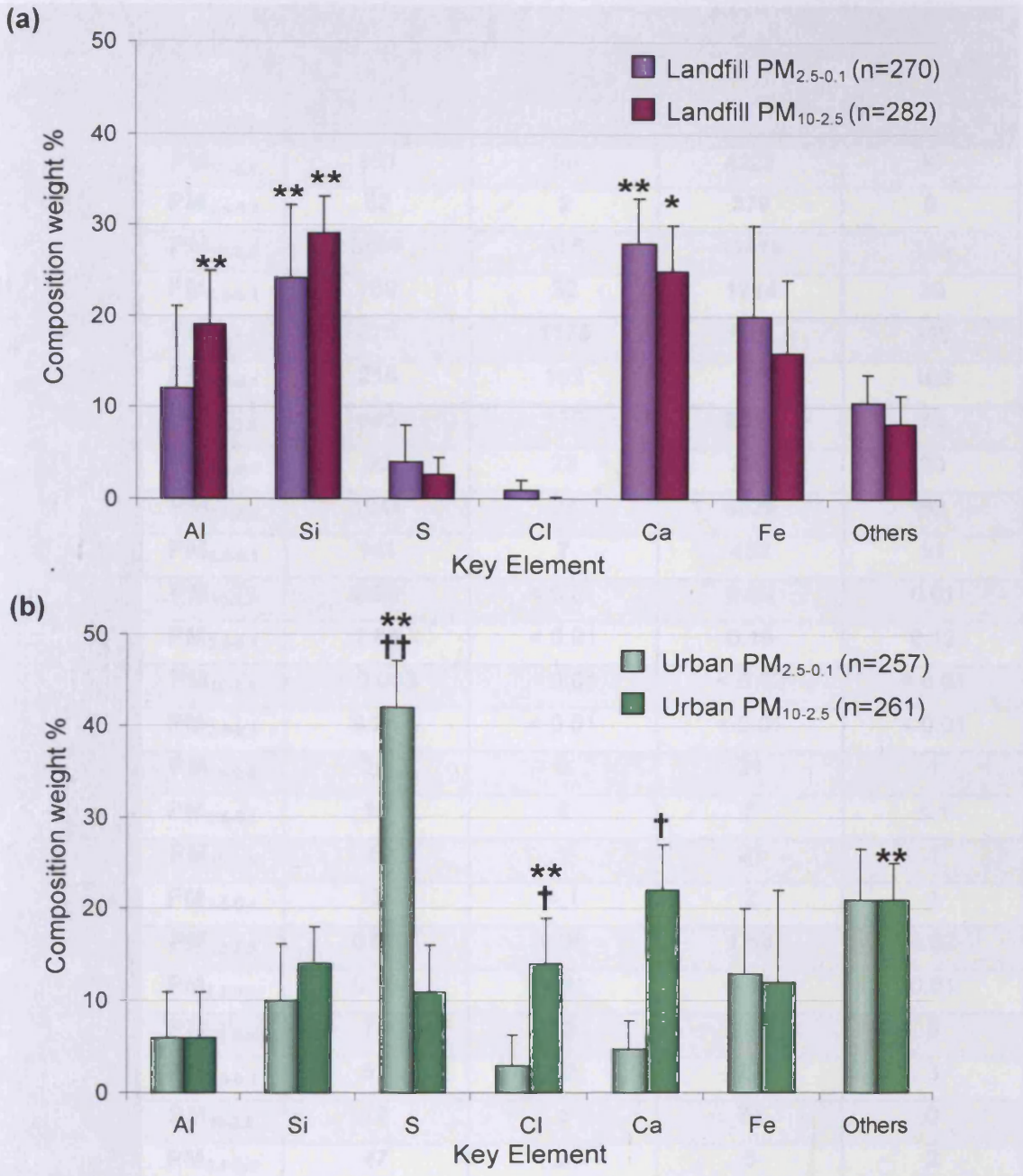

Figure 5.2: *FESEM-EDX 2008 analyses of PUF* ***(a)*** *landfill and* ***(b)*** *urban fractions collected in 2008 (mean ± stdev).* $PM_{2.5\text{-}0.1}$; $PM_{10\text{-}2.5}$; $PM_{2.5\text{-}0.1}$; $PM_{10\text{-}2.5}$. *There were inter-site differences. The landfill collection did not show size-dependent differences and was dominated by Si, Ca and Al in both fine and coarse modes. The urban 2008 sample exhibited a significant difference between the fine and coarse size modes, whereby a highly significant proportion of S was present in the fine fraction whilst Cl and Ca were more prevalent in the coarse fraction.* * $p \leq 0.05$, ** $p \leq 0.01$ *inter-site significance;* † $p \leq 0.05$, †† $p \leq 0.01$ *intra-site significance.*

Element	PM_{10} size fraction	Total Metal (ng/m^3)			
		2007		2008	
		Landfill	Urban	Landfill	Urban
Al	$PM_{10-2.5}$	831	59	**4227**	30
	$PM_{2.5-0.1}$	82	2	**379**	8
Ca	$PM_{10-2.5}$	3609	456	**11416**	174
	$PM_{2.5-0.1}$	769	32	**1214**	30
Na	$PM_{10-2.5}$	675	**1175**	618	340
	$PM_{2.5-0.1}$	**215**	162	128	103
Mg	$PM_{10-2.5}$	686	179	**2517**	70
	$PM_{2.5-0.1}$	96	23	**222**	20
Fe	$PM_{10-2.5}$	1044	97	**4739**	82
	$PM_{2.5-0.1}$	141	7	**432**	31
As (6)[a]	$PM_{10-2.5}$	0.29	< 0.01	**0.69**	0.01
	$PM_{2.5-0.1}$	**2.84**	< 0.01	0.19	0.12
Cd (5)[a]	$PM_{10-2.5}$	< 0.003	< 0.01	< 0.02	< 0.01
	$PM_{2.5-0.1}$	**0.24**	< 0.01	< 0.01	< 0.01
Cr (Cr^{6+} 0.2)[a]	$PM_{10-2.5}$	28	9	**51**	1
	$PM_{2.5-0.1}$	**17**	4	6	< 1
Cu	$PM_{10-2.5}$	8	1	**21**	1
	$PM_{2.5-0.1}$	**3**	< 1	2	1
Co	$PM_{10-2.5}$	0.50	0.06	**1.68**	0.02
	$PM_{2.5-0.1}$	0.16	0.01	**0.18**	0.01
Ni (20)[a]	$PM_{10-2.5}$	73	30	**184**	3
	$PM_{2.5-0.1}$	**57**	12	27	3
Pb (500)[a]	$PM_{10-2.5}$	17	1	**34**	0
	$PM_{2.5-0.1}$	**47**	2	6	2
V (50)[b]	$PM_{10-2.5}$	2.55	0.19	**4.83**	0
	$PM_{2.5-0.1}$	**1.96**	0.71	1.71	0.02
Mn (150)[a]	$PM_{10-2.5}$	68	4	**259**	3
	$PM_{2.5-0.1}$	17	1	**25**	1
Zn ($5mg/m^3$)[c]	$PM_{10-2.5}$	**49**	< 1	31	1
	$PM_{2.5-0.1}$	**159**	2	9	4

Table 5.3: *Average* ***total*** *$PM_{10-2.5}$ and $PM_{2.5-0.1}$ major and trace metal mass concentrations (ng/m^3) at landfill and urban sites (2007 and 2008). Highest value within each size range in bold font. Annual total metal limits [a]EPAQS (2008); [b]OSHA (2006a); [c]OSHA (2006b). Undetected species indicated by <LOD (See appendices A and B).*

Element	PM size fraction	Water-Soluble Metal (ng/m^3)			
		2007		2008	
		Landfill	Urban	Landfill	Urban
Al	$PM_{10-2.5}$	0	0	**146**	0
	$PM_{2.5-0.1}$	0	0	0	0
Ca	$PM_{10-2.5}$	2824	372	**3366**	186
	$PM_{2.5-0.1}$	**729**	32	604	18
Na	$PM_{10-2.5}$	**466**	903	n/a	390
	$PM_{2.5-0.1}$	**374**	129	58	66
Mg	$PM_{10-2.5}$	176	132	**221**	71
	$PM_{2.5-0.1}$	**80**	23	61	11
Fe	$PM_{10-2.5}$	20	3	**26**	2
	$PM_{2.5-0.1}$	24	7	5	**43**
As (6)[a]	$PM_{10-2.5}$	**0.48**	< 0.01	< 0.01	< 0.01
	$PM_{2.5-0.1}$	**3.30**	< 0.01	< 0.01	0.02
Cd (5)[a]	$PM_{10-2.5}$	< 0.01	< 0.01	< 0.01	< 0.01
	$PM_{2.5-0.1}$	**0.21**	< 0.01	< 0.01	< 0.01
Cr (Cr^{6+} 0.2)[a]	$PM_{10-2.5}$	0	0	1.28	0
	$PM_{2.5-0.1}$	0.47	**0.52**	0	0.08
Cu	$PM_{10-2.5}$	**1.3**	0.6	1.0	0.3
	$PM_{2.5-0.1}$	**3.2**	0	0.29	0.5
Co	$PM_{10-2.5}$	0	0	0	**0.004**
	$PM_{2.5-0.1}$	**0.054**	0.003	0	0.006
Ni (20)[a]	$PM_{10-2.5}$	< 0.10	< 0.10	< 0.10	< 0.10
	$PM_{2.5-0.1}$	**0.8**	0.40	< 0.10	0.14
Pb (500)[a]	$PM_{10-2.5}$	< 1	< 1	< 1	< 1
	$PM_{2.5-0.1}$	**23**	1	< 1	1
V (50)[b]	$PM_{10-2.5}$	< 0.10	< 0.10	< 0.10	< 0.10
	$PM_{2.5-0.1}$	**0.74**	0.50	< 0.10	0.12
Mn (150)[a]	$PM_{10-2.5}$	**9**	2	4	1
	$PM_{2.5-0.1}$	**11**	1	1	1
Zn (5mg/m^3)[c]	$PM_{10-2.5}$	1.5	< 0.1	1.0	**1.6**
	$PM_{2.5-0.1}$	**181**	4.1	0.1	4.1

Table 5.4: *Average* ***water-soluble*** *$PM_{10-2.5}$ and $PM_{2.5-0.1}$ major and trace metal mass concentrations (ng/m^3) at landfill and urban sites in 2007 and 2008. Highest value within each size range in bold font. Annual total metal limits [a]EPAQS (2008); [b]OSHA (2006a); [c]OSHA (2006b). Undetected species indicated by <LOD (See appendices A and B).*

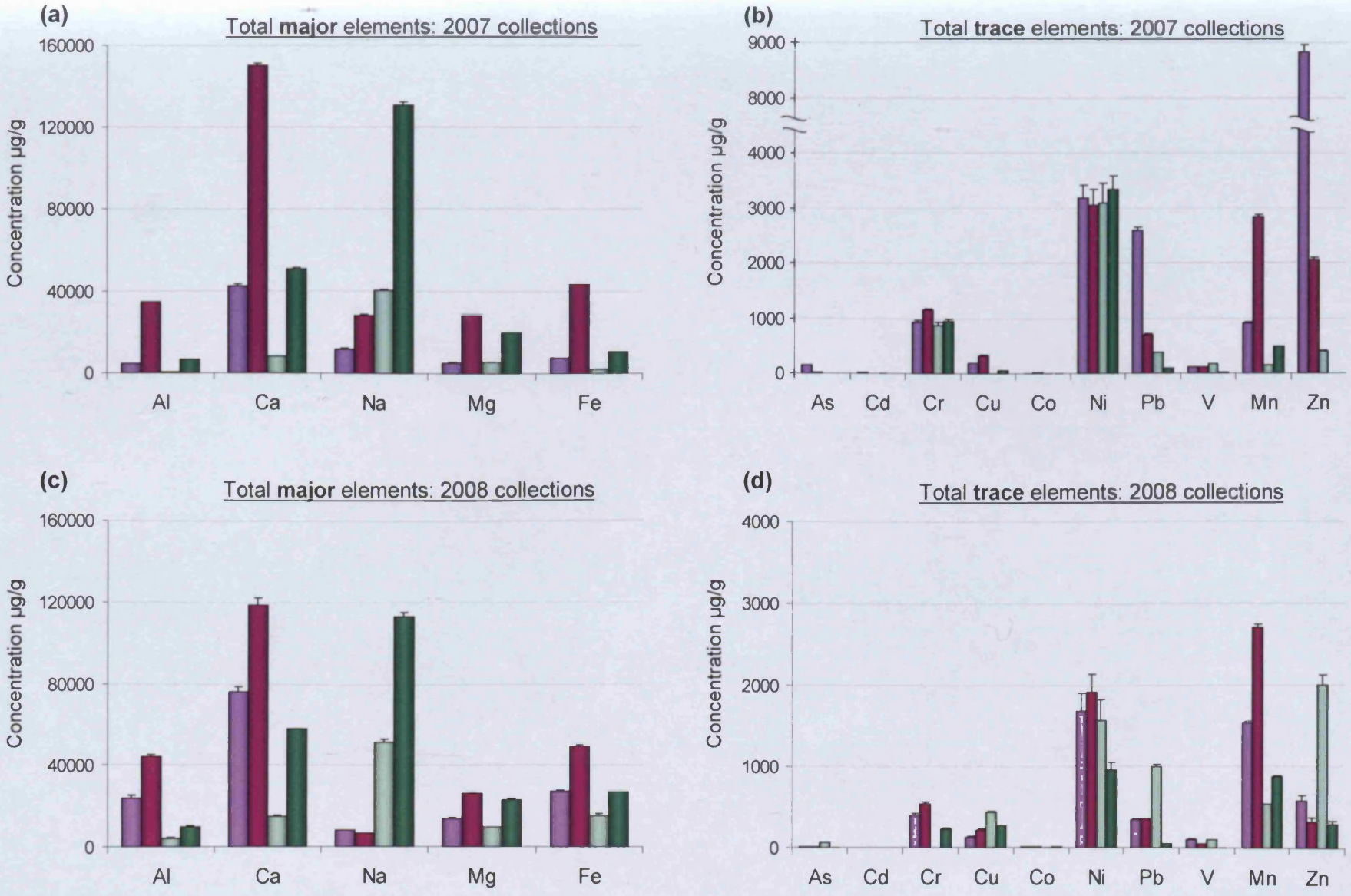

Figure 5.3: *Average elemental concentrations in µg/g of major and trace total metals in $PM_{2.5-0.1}$ and $PM_{10-2.5}$ whole fractions at landfill and urban sites in **(a - b)** 2007 and **(c – d)** 2008 PUF collections. Landfill $PM_{2.5-0.1}$; Landfill $PM_{10-2.5}$; Urban $PM_{2.5-0.1}$; Urban $PM_{10-2.5}$ (mean ± stdev). See Appendices A and B for tabulated data.*

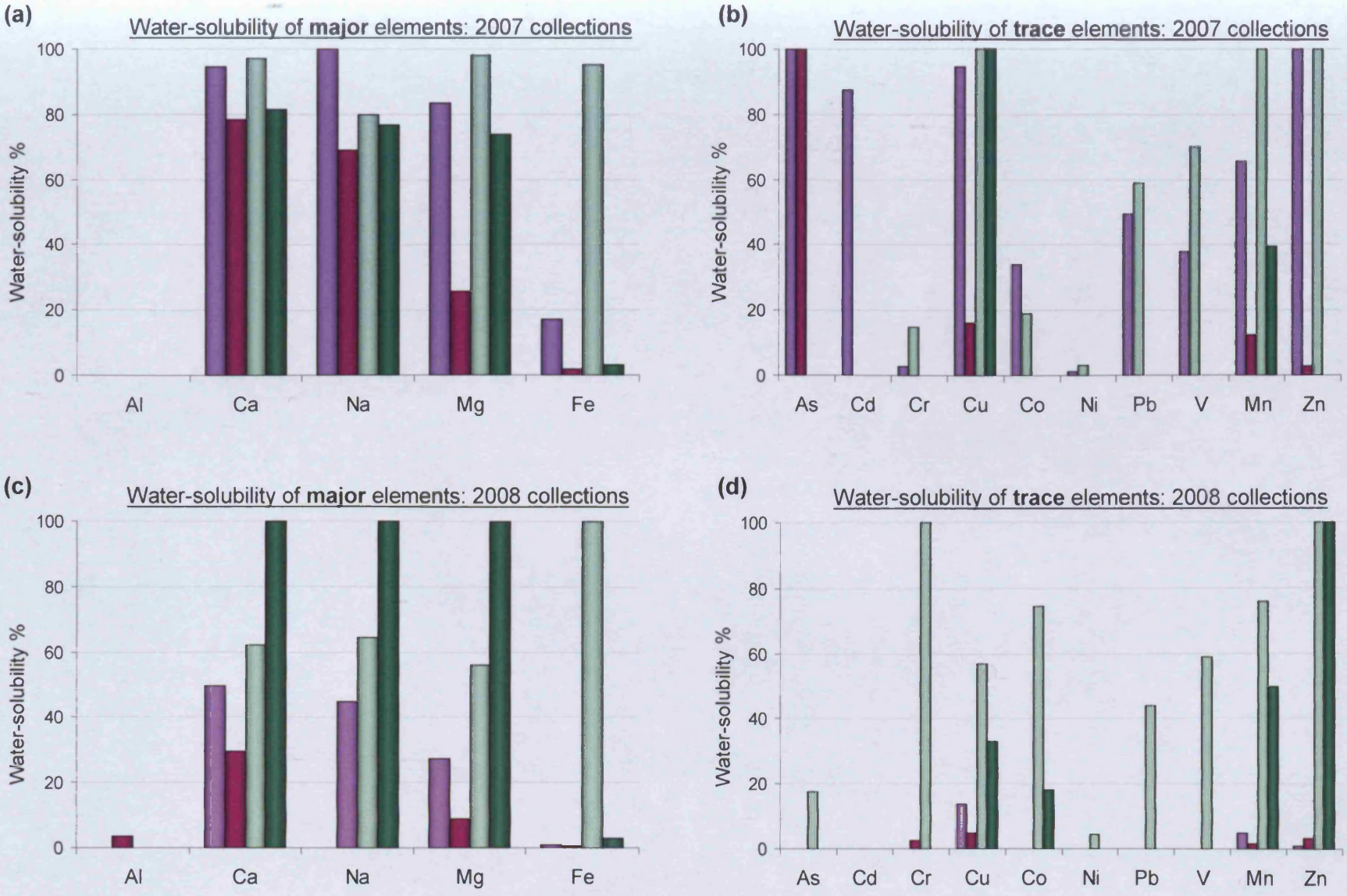

Figure 5.4: *Relative water-solubility percentage of major and trace metals in $PM_{2.5-0.1}$ and $PM_{10-2.5}$ aqueous fractions of landfill and urban collections in* ***(a - b)*** *2007 and* ***(c – d)*** *2008 PUF collections.* ■ *Landfill $PM_{10-2.5}$;* ■ *Landfill $PM_{2.5-0.1}$;* ■ *Urban $PM_{10-2.5}$;* ■ *Landfill $PM_{2.5-0.1}$. See Appendices A and B for tabulated data.*

The particulate metal concentrations were reported after solvent blank correction. The measured instrumental limits of detection (LOD) for all elements were calculated as being three times the standard deviation of the mean of at least six measurements of a solvent blank (Hodson, 2002), and were 2-8% of the lowest sample concentration. Metal concentrations in the procedural particulate blank, CB M120, run in parallel with total PM sample digestion, were below the LOD for all elements.

5.3.2.1 PM_{10} Total Airborne Mass Concentrations

Airborne mass concentrations (ng/m^3) were reported for the total (representing the insoluble and water-soluble portions) and water soluble metal contents (Tables 5.3 and 5.4), along with recommended regulatory standards for elements of toxicological significance (EPAQS, 2008; OSHA, 2006a,b). The only elements to exceed these values were Ni, Cr and Mn. Nickel exceeded the standard limit in all samples, except in the urban 2008 collection. The Cr water-soluble data indicated both urban and landfill 2007 $PM_{2.5\text{-}0.1}$ samples and the landfill 2008 $PM_{10\text{-}2.5}$ fraction, may have been above the guideline value (assuming soluble Cr was present as Cr^{6+}; Table 5.4). Manganese was above the guideline concentration in the landfill 2008 coarse sample only.

In comparison to the urban collections, the landfill collections tended to yield the largest mass concentrations for major and trace elements in both size fractions. The sole exception to this trend was Na, which was found at the highest concentration in the urban coarse mode of 2007. Aluminium, Ca, Mg and Fe were several-fold more concentrated in the landfill 2008 collection than any other particulate sampling period in this study. There was a highly significant amount of Zn in the landfill 2007 sample compared to any other sample.

5.3.2.2 PM_{10} Total Metal Concentrations

The metals Al, Ca, Mg and Fe were analysed due to their association with geological material, and were present as major PM components.

5.3.2.2.1 PM_{10} Collections 2007

Both landfill and urban 2007 coarse samples contained a higher proportion of the major elements (Al, Ca, Na, Mg, Fe) versus their corresponding fine fractions. Calcium was the dominant major element in landfill $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$, whereas Na was the major element in urban PM_{10}, at concentrations far higher than those seen in any of the corresponding landfill samples (Figure 5.3.a). Although Ni was a significant signal in all samples, there were no significant site- or size-dependent differences. All trace metals were present below 4000µg/g, except Zn in landfill $PM_{2.5\text{-}0.1}$, which was detected at 8835µg/g (Figure 5.3.b). Zinc and Pb were the predominant trace elements of the landfill $PM_{2.5\text{-}0.1}$, whilst Mn and Cr were significant contributors to landfill $PM_{10\text{-}2.5}$.

5.3.2.2.2 PM_{10} Collections 2008

Calcium was the dominant element of the landfill PM; double the amount of Ca, compared to the previous year's landfill 2007 PM_{10} collection, was detected in both modes (Figure 5.3.c; $p \leq 0.05$). Sodium was the dominant urban PM_{10} metal. Nearly all major metals were preferentially partitioned into the coarse mode; however, the opposite was true for the landfill 2008 Na content, whereby Na was present at a higher concentration in the fine fraction (6440µg/g and 8000µg/g, respectively). This was not a statistically significant difference ($p > 0.05$).

All trace elements were present below 3000µg/g. Of interest, the landfill 2008 $PM_{2.5\text{-}0.1}$ Zn and Pb signals were diminished to less than 10% and 13% of the exceptionally high Zn and Pb concentrations detected in the previous year's landfill $PM_{2.5\text{-}0.1}$ sampling (Section 5.3.2.2.1). With the exception of Ni, Mn was the predominant trace element in both landfill $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ fractions, as well as the urban $PM_{10\text{-}2.5}$ sample. In contrast, Zn and Pb were the principal species in the urban $PM_{2.5\text{-}0.1}$ fraction.

5.3.2.3 PM_{10} Water-soluble Metal Concentrations

Owing to differential LODs, elements which were undetectable in the total particulate digests, but which were quantifiable in the water-soluble fraction were reported as 100% soluble.

5.3.2.3.1 PM_{10} Collections 2007

Of the major elements, only Al and Fe showed very limited solubility. However, Fe was 95% soluble in the urban fine sample (Figure 5.4.a). The landfill coarse fraction also exhibited low solubility for Mg when compared to either the fine fraction, or the urban coarse fraction. Regardless of sampling location, the coarse samples displayed the lowest water-solubility across nearly all elements (with the exception of urban $PM_{10-2.5}$ Cu and Mn). In stark contrast, the fine fractions of landfill and urban 2007 collections displayed very high water-solubility for Cd, Cu, Co, Pb, V, Mn and Zn (Figure 5.4.b). Of the trace elements, Ni and Cr appeared to be the least water-soluble.

5.3.2.3.2 PM_{10} Collections 2008

In comparison to the landfill PM_{10} 2007 collection, there was a widespread reduction in the water-solubility of the major elements in both landfill 2008 PM_{10} fractions (Figure 5.4.c). The urban 2008 coarse and fine fractions exhibited a consistent high degree of major element solubility – the high solubility of Fe in the urban $PM_{2.5-0.1}$ was observed year-on-year.

The most distinct difference between the landfill and urban 2008 samples was seen with the solubility of trace elemental species. Neither landfill $PM_{10-2.5}$ nor $PM_{2.5-0.1}$ were water-soluble in comparison with the corresponding urban fractions (Figure 5.4.d). The Zn content of both urban $PM_{10-2.5}$ and $PM_{2.5-0.1}$ was reported as 100% water-soluble. The urban $PM_{10-2.5}$ fraction demonstrated a contribution from water-soluble Cu, Co and Mn. The fine fraction appeared to possess the highest proportion of water-soluble Cu, Co, Pb, V and Mn. The low solubility of landfill $PM_{2.5-0.1}$ was in stark contrast to the previous year's collection.

5.3.3 IC

The water – soluble fractions of landfill and urban collections were analysed by ion exchange chromatography. The results were grouped into the $PM_{10-2.5}$ and $PM_{2.5-0.1}$ size fractions, and presented as both percentage composition of sample mass, and airborne mass concentrations (Table 5.5; Figure 5.5). The

landfill PM_{10} 2007 collection tended to present the highest mass concentrations of all three major anions (Cl^-, SO_4^{2-} and NO_3^-) measured in either $PM_{2.5\text{-}0.1}$ or $PM_{10\text{-}2.5}$ fraction. The sole exception was the association of Cl^- with urban 2007 $PM_{10\text{-}2.5}$ (Table 5.5).

Both the landfill and urban 2008 $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ fractions exhibited lower airborne anion mass concentrations, compared to the samples collected at the same site in the previous year (Table 5.5). This year-on-year decline in anion airborne mass concentrations was greater in the landfill collection, than in the urban collection.

Anion ($\mu g/m^3$)	PM_{10} size fraction	Sample collection			
		2007		2008	
		Landfill	Urban	Landfill	Urban
Cl^-	$PM_{10\text{-}2.5}$	0.57 ± 0.00	**1.07 ± 0.18**	0	0.39 ± 0.12
	$PM_{2.5\text{-}0.1}$	**0.64 ± 0.00**	0	0.10 ± 0.16	0
SO_4^{2-}	$PM_{10\text{-}2.5}$	**1.38 ± 0.23**	0.26 ± 0.00	0.92 ± 0.00	0.13 ± 0.03
	$PM_{2.5\text{-}0.1}$	**2.94 ± 0.60**	0.73 ± 0.00	0.44 ± 0.09	0.33 ± 0.07
NO_3^-	$PM_{10\text{-}2.5}$	**1.84 ± 0.48**	0.78 ± 0.19	0	0.65 ± 0.18
	$PM_{2.5\text{-}0.1}$	**9.90 ± 1.87**	0.19 ± 0.01	0.12 ± 0.03	0.12 ± 0.02

Table 5.5: *Airborne PM_{10} mass concentrations of major $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ anion species (mean ± stdev). The highest value within each size range is shown in bold font.*

The total water-soluble anion content of PM_{10} was generally more concentrated in the $PM_{2.5\text{-}0.1}$ fractions, with the exception of the urban 2008 PM_{10}. This was exemplified by the percentage composition (Figure 5.5). The $PM_{10\text{-}2.5}$ samples exhibited the highest Cl^- contents; notably, these were at the urban locations (Figure 5.5.a). Sulphate and NO_3^- were ubiquitous and contributed to the sample mass in all PM_{10} samples; however, chloride was not detected in urban $PM_{2.5\text{-}0.1}$ samples.

Anion levels of the landfill collections were widely variable, with the landfill

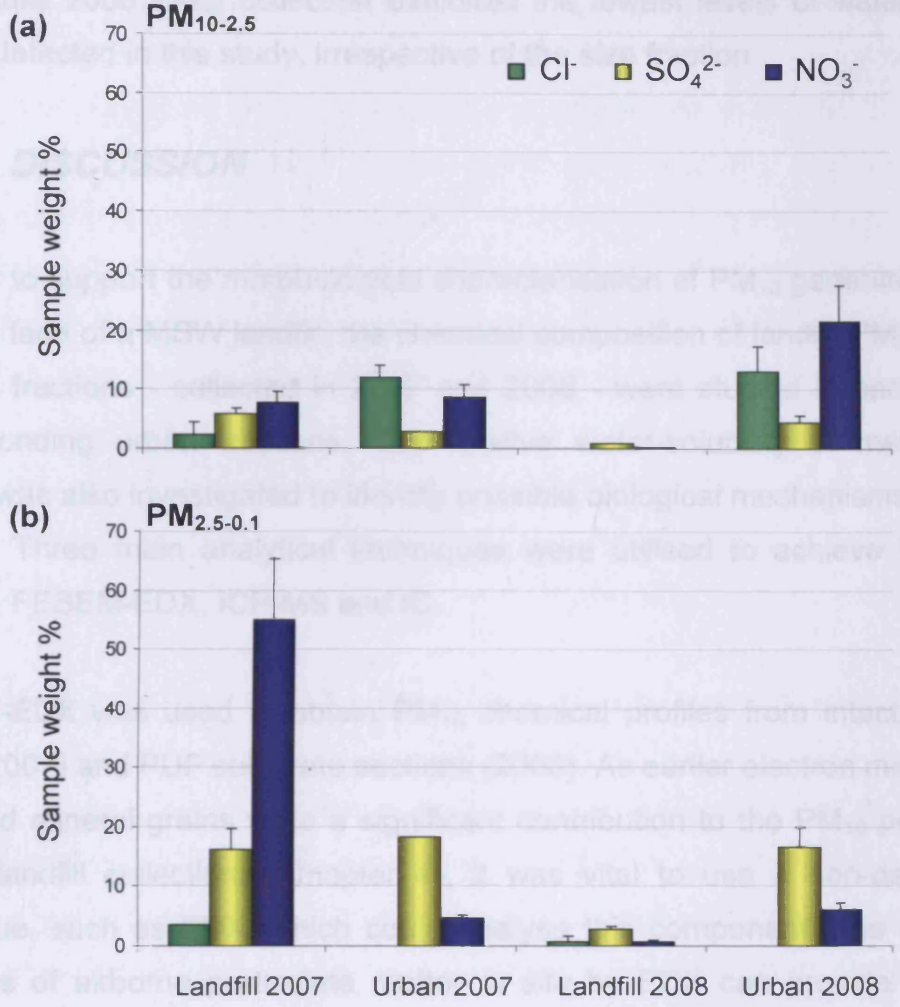

Figure 5.5: *Ion chromatography for the evaluation of chloride, sulphate and nitrate in* ***(a)*** *$PM_{10-2.5}$ and* ***(b)*** *$PM_{2.5-0.1}$ fractions of landfill and urban PM_{10} collected in 2007 and 2008 (mean ± stdev). Chloride was preferentially fractionated into the $PM_{10-2.5}$ mode, whilst sulphate and nitrates dominated in the $PM_{2.5-0.1}$ mode.*

$PM_{2.5-0.1}$ samples yielding the highest and lowest nitrate proportions observed in the study - 55% of sample weight in 2007 - whilst the corresponding 2008 collection contained just a trace level of nitrate 0.7% contribution. $PM_{2.5-0.1}$ sulphate was present at relatively constant levels of 16-18% of sample mass in both 2007 and 2008 urban fine samples, and this was similar to the 16% sulphate content seen in the landfill 2007 $PM_{2.5-0.1}$ sample (Figure 5.5.b); nevertheless, the landfill 2008 fine fraction contained the least sulphate ($p \leq 0.05$).

The landfill 2008 PM_{10} collection exhibited the lowest levels of water-soluble anions detected in this study, irrespective of the size fraction.

5.4 DISCUSSION

In order to support the morphological characterisation of PM_{10} generated at the working face of a MSW landfill, the chemical composition of landfill $PM_{2.5-0.1}$ and $PM_{10-2.5}$ fractions - collected in 2007 and 2008 - were studied in parallel with corresponding urban fractions. The relative water-solubility of metals and anions was also investigated to identify possible biological mechanisms of PM_{10} toxicity. Three main analytical techniques were utilised to achieve this aim; namely, FESEM-EDX, ICP-MS and IC.

FESEM-EDX was used to obtain PM_{10} chemical profiles from intact Negretti filters (2007) and PUF substrate sections (2008). As earlier electron microscopy indicated mineral grains were a significant contribution to the PM_{10} population of the landfill collections (Chapter 4), it was vital to use a non-destructive technique, such as EDX, which could analyse this component. The chemical analyses of airborne particulate matter *in situ* by EDX can provide valuable information on important airborne particulate elemental trends (EPA, 2002; Paoletti *et al.*, 2002; Mogo *et al.*, 2005). Although it can not provide quantitative chemical data due to the inaccuracies of SEM (discussed below), the technique can reliably detect mineral matter, such as silicates and fly ash, due to the high atomic number contrast. The BSE signal (back scatter electrons reflected from the interior of the sample) is largely determined by the atomic number of the element present in the solid sample. Hence, low atomic weight elements show poor contrast effects, making the identification of nitrogenous (e.g. ammonium sulphate) and organic compounds very difficult against the grey background (Pratesi *et al.*, 2007). The use of polycarbonate filters or polyurethane foam substrates for PM_{10} accumulation, rather than quartz filters, meant it was possible to assign Si proportions by FESEM-EDX. This was particularly relevant in this investigation, as Si was subsequently discovered to be the dominant species of landfill PM_{10} in 2008.

The absence of an 'organics estimate' was regrettable, as the carbonaceous cores of combustion-derived particles are known to adsorb a plethora of reactive metal species and hydrocarbon anions (de Kok *et al.*, 2006). This led to bias against such material during analysis. In addition, the complicated 'rough' topology of particles meant that in reality, the PM were far from the ideal flat surface which was assumed by EDX analyses algorithms. Particles were often smaller than the area rastered by the backscatter electron (BSE) beam, meaning X-rays emitted from adjacent PM could influence the spectra (Paoletti *et al.*, 2002). Although the use of EDX on such particulate samples was subject to systemic errors, extensive and careful selection of filter areas for analysis minimised these inaccuracies; this revealed the important PM types (Moreno *et al.*, 2003; Tasić *et al.*, 2006).

ICP-MS was used to analyse HVCI samples for total and water-soluble metal content. Although this was a highly sensitive technique, and able to quantify elements at the µg/g level, it was unable to confirm the presence of Si due to the omission of HF from the solvent in microwave sample preparation. An additional confounder may have arisen due to instrumental contamination from the nickel sampling and skimmer cones (Heilmann *et al.*, 2009; McDonald, I., personal communication, 2009). Nonetheless, major elements (Al, Ca, Na, Mg, Fe) and trace metals (As, Cd, Cr, Cu, Mo, Ni, Pb, V, Fe, Mn, Zn) were quantified, expressed as a component of the PM_{10} fractions (µg/g; in order to discuss individual metal contributions to the sample) and related to pollution mass concentrations (ng/m^3).

Converting absolute elemental concentrations (µg/g) into mass concentrations (ng/m^3), allowed for discussion in terms of potential population exposure via inhalation. Very few elements were measured at concentrations exceeding air quality standards, with the exception of Ni (which may have been due to instrumental contamination as postulated above), Mn (landfill 2008 $PM_{10\text{-}2.5}$) and Cr (landfill and urban 2007 $PM_{2.5\text{-}0.1}$, and landfill 2008 $PM_{10\text{-}2.5}$). Although the water-soluble fraction of the landfill PM_{10} samples had been investigated here, it was not possible to confirm this was the *in vivo* physiologically bioavailable fraction of metals. The respiratory tract lining fluid, being a complex

mixture, contains chelators, antioxidants and is maintained at an acidic pH; all of which affect the leaching and mobility of metals from inhaled and retained PM_{10} (Mudway *et al.*, 2004). Nonetheless, water-solubility is considered to be an indication of loose chemical association (Dos Santos *et al.*, 2009), and meaningful inferences about potential metal bioavailability can be drawn from the levels of dissociation into aqueous matrices. Therefore, although the concentration of a given metal may be elevated in the total PM_{10} sample, the element may be relatively immobilised in the crystalline network of mineral matter and would be present at a much lower concentration in the water-soluble extract. The water-solublity of the transition metals - Fe, V, Cu, Ni, Co, Cr - and Pb was of particular interest, as these have been linked to the formation of ROS and alteration of cellular homeostasis (Stohs and Bagchi, 1995; Valko *et al.*, 2007; Valavanidis *et al.*, 2008).

The identification of apparently 100% soluble samples was likely to be a reflection of the more complex matrix produced by the microwave digestion, which resulted in a higher inherent background, versus the simple agitation and acidification procedure of obtaining a water-soluble PM_{10} extract (Karthikeyan *et al.*, 2006). This may also have been due to problems in analysing highly soluble metals or heterogenous samples containing metal rich "nuggets" distorting average values (Merolla, 2005). Interestingly, an example of this was seen with the redox-active metal, Cu, which was below the LOD in the urban 2007 total $PM_{2.5\text{-}0.1}$ particulate digest (Figure 5.3.b), but was detectable in the accompanying water-soluble extract (Figure 5.4.b, ~ 100% solubility). Other metals which exhibited this pattern were the urban 2007 $PM_{10\text{-}2.5}$ Zn and urban 2008 $PM_{2.5\text{-}0.1}$ Cr. It might have been pertinent that the trend of increased trace element aqueous solubility only occurred in the urban-sourced samples.

Ion Chromatography was used to analyse the water-soluble component of the landfill and urban PM_{10} for the major particulate-associated acidic anions (Cl^-, SO_4^{2-}, NO_3^-). These are the acidic anions most commonly detected in airborne PM (Harrison and Yin, 2000; Wang *et al.*, 2005), and thus lend the samples to comparison with other studies. The acidic properties of fine particulates have been correlated to adverse health effects (e.g. increased airway resistance;

Lippmann and Thurston, 1996), yet the biological mechanisms remain unclear. As these anions are known to form salts with metal components of PM_{10}, it was postulated this reaction increased their mobility, surface reactivity and hygroscopic nature of the airborne particulates (Ocksay *et al.*, 2006).

5.4.1 PM_{10} COLLECTIONS 2007

Negretti filters were used for the FESEM-EDX analysis due to the complete extraction of the parallel PUF collection for bioreactivity, ICP-MS and IC analyses. FESEM-EDX analysis revealed the landfill 2007 PM_{10} was composed of a greater proportion of aluminosilicates than the corresponding urban PM_{10} 2007 sample. This was in good agreement with the morphological characterisation, whereby mineral grains were shown to dominate the landfill particulate classification, indicating a geological source (Chapter 4). The low solubility of Al and Fe indicated these were bound in strong, covalent associations, supporting their presence in aluminosilicate matrices (Desboeufs *et al.*, 2005). The proportion of Al detected by FESEM-EDX (Negretti) in landfill PM_{10} was greater than the concentration indicated by the ICP-MS (HVCI) data. Although incomplete release of elements from the silicate matrix during ICP-MS preparation was a possibility, this is not likely to have been a major interference due to sample dissociation in the plasma source (Karthikeyan *et al.*, 2006). This difference in Al may be due to a confounding effect of the different PM_{10} populations obtained from the Negretti versus the HVCI collector: it was unfortunate that FESEM-EDX could not be performed on HVCI 2007 substrates.

The relative solubility of the trace metals in fine fractions of both landfill and urban 2007 collections indicated that the smallest particulates from these samples were likely to have arisen from carbonaceous cores which became adsorbed with metals and salts, unlike mineral aluminosilicate particles (Desboeufs *et al.*, 2005). In particular, the solubility of the redox-active metals Cd, Cu, Co, V, Mn, Pb and Zn was elevated in both landfill and urban 2007 fine fractions, suggesting these may be highly mobile in the respiratory tract. The most likely contributor of these elements would have been anthropogenic

sources of combustion pollution, primarily vehicle exhaust emissions (Donaldson *et al.*, 2005; Nelson *et al.*, 2008). Zinc, Cd and Cu may also have arisen from tyre and brake wear (WHO, 2002; Fuge, 2005).

Although strikingly higher than other samples, the elevated total Zn particle airborne mass concentration seen in the landfill 2007 $PM_{2.5\text{-}0.1}$ fraction (159ng/m^3), did not exceed the regulatory standard, and was in a similar range to urban airborne $PM_{2.5\text{-}0.1}$ mass concentrations published in the current literature. Dos Santos *et al.*, (2009) reported $PM_{2.5}$ Zn concentrations of 77-234ng/m^3 (Buenos Aires); whereas Heal *et al.*, (2005) reported $PM_{2.5}$ Zn levels of 7.49ng/m^3 (Edinburgh) that was similar to the smaller mass concentrations exhibited by the urban 2007 $PM_{2.5\text{-}0.1}$ sample. Despite Zn not being a true transition metal (it possesses a filled *d*-orbital shell), it has been linked to oxidative stress (Frampton *et al.*, 1999; Valko *et al.*, 2007). The solubility of silicated Zn has been reported to be no greater than 20% (Desboeufs *et al.*, 2005), however, the discovery of a large soluble Zn (10064µg/g) and Pb (1280µg/g) content at the landfill, yet a poorly soluble Fe (1350µg/g) component, may have implications upon the PM_{10} bioreactivity. Gutiérrez-Castillo *et al.*, (2005) reported soluble PM_{10} Zn and Pb at 2109µg/g and 8µg/g, respectively (Mexico City). In comparison, Heal *et al.*, (2005) reported soluble $PM_{2.5\text{-}0.1}$ Zn and Pb concentrations at approximately 400 and 450µg/g respectively (Edinburgh); both significantly lower than the concentrations determined in the landfill 2007 $PM_{2.5\text{-}0.1}$.

Both Fe and Mn are known to be common transition elements in crustal PM_{10}, as they are the main substitute elements in aluminosilicate minerals, and were shown to contribute more to the $PM_{10\text{-}2.5}$ total metal profile. However, both were more soluble in the $PM_{2.5\text{-}0.1}$ fraction at both sampling sites (Figure 5.4). Unlike either coarse or fine landfill 2007 samples, Fe was highly water-soluble in the urban $PM_{2.5\text{-}0.1}$, pointing to the presence of varying particle types at the study locations. Despite Fe being a known redox-active metal, the absence of significant water-soluble Fe in landfill PM_{10} inferred the metal was strongly bound within the particulate aluminosilicate matrix at the waste site, and perhaps unavailable to catalyse redox reactions. Iron is known to form soluble

salts with sulphate (Gutírrez-Castillo *et al.*, 2005); however, as both landfill and urban $PM_{2.5-0.1}$ sulphate anion percentage values were not significantly different from each other, it seemed plausible to suggest that urban Fe was present as other additional labile species.

Nickel was a significant contributor to the total trace elemental content: its detection may have been distorted by inaccuracies of Ni quantification due to the Ni cones present at the Thermo X7 interface. The low solubility of Ni and Cr alluded to their immobilised presence within crustal material.

Ion chromatography indicated landfill $PM_{10-2.5}$ and $PM_{2.5-0.1}$ chloride percentage values were not significantly different from each other; yet there was a greater chloride percentage contribution to the urban $PM_{10-2.5}$ fraction. This was a surprising finding in light of the proximity of the landfill to the Severn Estuary, as sea salt would have been an expected contributor to landfill PM_{10}, but this result was in agreement with the morphological profile (Chapter 4). The landfill $PM_{2.5-0.1}$ sample exhibited a doubling in the airborne mass concentration of nitrate compared to values generally quoted in the urban PM literature (0.35 to 3.5μg/m^3: various cities, Godoy *et al.*, 2009), but was similar to the NO_3^- levels in Beijing air (11μg/m^3: non-dust storm, Wang *et al.*, 2005). Although sea salt is a known contributor to particulate nitrates, they would have fallen predominantly into the coarse fraction (Harrison *et al.*, 2004), and hence the reason for the high landfill $PM_{2.5-0.1}$ 2007 nitrate was unknown.

5.4.2 PM_{10} Collections 2008

Following morphological investigation of PM_{10} collected in 2007 (Chapter 4), it was decided to carry out subsequent FESEM-EDX analyses on HVCI (PUF) substrate sections in preference to Negretti filters. This was considered a method improvement, as many investigators advocate the use of a single PM population for parallel physicochemical and toxicological characterisation (Greenwell *et al.*, 2002; Jones *et al.*, 2002; Sandström *et al.*, 2005).

The FESEM-EDX analysis revealed the geological elements Al, Ca, Si and Fe were the main contributors to landfill $PM_{10-2.5}$ and $PM_{2.5-0.1}$ fractions. The lack of any statistical difference between the landfill PM_{10} size fractions inferred a relatively homogenous mineral PM_{10} population at the landfill site. In contrast, the significant particle size-dependent differences in Ca, S and Cl in the urban fractions indicated a heterogeneous particle composition at the urban location.

Although ICP-MS revealed the landfill 2008 $PM_{10-2.5}$ and $PM_{2.5-0.1}$ ranked higher than the urban collection, in terms of mass concentrations for all transition and heavy metals; these were also the least soluble PM sample collected, according to ICP-MS and IC. The airborne mass concentrations of landfill Al and Ca were unusually elevated in comparison with the current literature, which generally report $PM_{10-2.5}$ Al mass concentrations of 37 – 2130ng/m^3 (Godoy *et al.*, 2009), whilst Dos Santos *et al.*, (2009) reported $PM_{10-2.5}$ Ca concentrations of up to 4884ng/m^3 (Buenos Aires). The particulate concentration of the important geological elements (Al, Ca, Mg and Fe and Mn) in the landfill 2008 collection correlated well with the previous year's PM_{10}, but this was not repeated in the trace elemental content, which showed significantly decreased transition metal content in 2008.

Interestingly, the urban $PM_{2.5-0.1}$ contained a large proportion of soluble Cu, Co, Pb, V, Mn and Zn versus the equivalent landfill size fraction, which was poorly soluble. Although it was not possible to draw definitive temporal conclusions based on the limited number of samples obtained during this study, it seemed sensible to suggest a greater chemical variation at the landfill $PM_{2.5-0.1}$ than at the urban location.

As mentioned earlier, anions may be used as indicators of potential transition metal mobility, and it was therefore interesting to note the similar percentage contributions of sulphate seen in all $PM_{2.5-0.1}$ fractions, with the exception of the landfill 2008 collection, where sulphate was significantly decreased. The total anion content of the landfill 2008 collection amounted to just 5% of the PM_{10} mass content - the lowest concentration of soluble salts in this study.

The intense dust episodes, due to the high levels of soil entrainment from vehicular movements between the weighbridge and the working face during landfill 2008 sampling, were believed to be mainly of insoluble crustal material; whereas combustion-derived particles often have soluble sulphates, transition metals and organics adsorbed to their surface (de Kok *et al.*, 2006). Hence, it seemed likely this anthropogenic particulate type dominated the chemistry of the urban 2008 collection to a greater extent than the landfill 2008 collection.

5.5 CONCLUSIONS

In summary, landfill PM_{10} collections exhibited significant year-on-year chemical variation, whilst the elemental and anionic composition of urban PM_{10} remained relatively constant. Geological elements (Al, Si, Ca, Mn, Fe) were elevated at the landfill in both 2007 and 2008, however, the initial (2007) landfill PM_{10} sampling exhibited far greater metal solubility, albeit lower mass concentrations than the later (2008) landfill PM_{10} collection. This was most pertinent to the $PM_{2.5\text{-}0.1}$ fraction, which would have remained airborne for longer than the $PM_{10\text{-}2.5}$ fraction.

The landfill $PM_{10\text{-}2.5}$ fractions exhibited consistently poor metal solubility. In general, the landfill $PM_{2.5\text{-}0.1}$ contained a highly significant amount of soluble Zn, Pb and NO_3^- in 2007, indicating a greater anthropogenic influence to the initial landfill PM_{10} sample. In contrast, the later 2008 landfill collection appeared to comprise largely insoluble mineral particulates in both the coarse and fine fractions, suggesting metals bound within crustal material would not be bioavailable.

Although the landfill 2008 campaign displayed the highest airborne mass metal concentrations, this investigation also emphasised the importance of evaluating chemical composition by airborne mass concentrations (ng/m^3), as particulate masses (μg/g), fail to provide a complete insight into the toxicological potential of PM_{10}.

CHAPTER 6

BIOREACTIVITY OF PARTICULATE MATTER

6.1 INTRODUCTION

Following the physicochemical characterisation of landfill PM_{10}, it was necessary to ascertain the hazard of the two different landfill PM_{10} collections. This was compared to urban PM_{10} bioreactivity. As shown in Chapters 4 and 5, the composition of airborne PM can vary widely year-on-year; hence it was necessary to investigate how these differences affected the potential toxicity of landfill PM_{10}. It has been hypothesised by many investigators, that much of the toxicity of PM_{10} is related to its capacity to induce systematic oxidative stress. Hence, the research aim of this section was to evaluate the *in vitro* bioreactivity of the $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ fractions by the redox-sensitive plasmid scission assay (PSA), and exposure upon the EpiAirway™ human tracheobronchial model.

The bioreactivity of $PM_{2.5\text{-}0.1}$ (fine), $PM_{10\text{-}2.5}$ (coarse) and composite PM_{10} samples were evaluated by the PSA. The PM samples were then fractionated into their water-soluble and insoluble (biopersistent) fractions and re-evaluated for pro-oxidant activity. The modulating effects of metal chelators (EDTA, DTPA or DES) and an in-house surrogate epithelial lining fluid (SELF) were also assessed.

Although the PSA is an easily adapted technique, it remains a basic tool in the elucidation of PM ROS-generating capacity, and can not be used to infer potential genotoxicity or adverse *in vivo effects*. Unlike nuclear or mitochondrial DNA which is compartmentalised and under protective mechanisms, the plasmid DNA of the PSA is a naked molecule, purified from bacteria, and thus, lacks any cellular regulation. Subsequent to the identification of oxidative PM samples from the PSA, it was prudent to advance the investigation onto the human-derived tracheobronchial cell culture stage.

Since the entire airway epithelium is a primary site of PM_{10} contact, different pulmonary cell lines (macrophages, alveolar type II cells) as well as extra-pulmonary epithelial cell lines (HepG2: liver, Caco-3: colorectal epithelial) have

been utilised as *in vitro* models (de Kok *et al.*, 2006). The EpiAirway™ system has been used in a variety of *in vitro* studies as a surrogate for animal studies: a wide range of compounds have been tested for their toxicity and inflammation (Agu *et al.*, 2006; Balharry *et al.*, 2008; Sexton *et al.*, 2008). The model has been commercially available since 1998 and has been described as "normal, human derived tracheal/bronchial epthelial cells, which have been cultured to form a highly differentiated model of the human airway" (BéruBé *et al.*, 2006). The EpiAirway™ tissue is metabolically and mitotically alive; protective mucus is secreted from goblet cells, cilia beat and cells form tight junctions. It is a highly differentiated culture and since it is not developed from lung tumours or diseased tissue, the cells are genotypically and phenotypically more representative of normal epithelia than other commonly used *in vitro* pulmonary cell systems, such as Calu-3 and A549 (carcinoma-derived) or BEAS-2B (viral-transformed) epithelial lines (BéruBé *et al.*, 2009).

The characterisation of the genetic responses of cells and tissues to a variety of toxins and chemical species is known as "toxicogenomics". Various methods have been used to assess changes in gene expression, such as quantitative polymerase chain reaction (qPCR) and Northern blotting. There are, however, problems in selecting individual genes to investigate, as important links may be missed between different gene activities. Recent technologies known as hybridisation arrays have addressed this problem, as they are used to analyse global gene expression, enabling simultaneous comparison of whole complementary DNA (cDNA) populations (Sobek *et al.*, 2006).

This microarray technology is based on hybridisation by base-pairing of the cRNA target (complementary RNA representing messenger RNA (mRNA) from study samples) to the probe (cDNA that is fixed, as 60-mer oligonucleotide spots on nylon membranes). The arrays are scanned and hybridisation signals of the spots are quantified by suitable image analysis software. Such hybridisation arrays take advantage of the high affinity and specificity of nucleotide complementary base-pairing. The technology is not without its drawbacks - arrays only measure relative, not absolute, levels of mRNA expression and changes in RNA do not necessarily correlate to equivalent

changes in protein expression (Pennie *et al.*, 2000). Nonetheless, for the majority of genes, changes in mRNA abundance are related to changes in protein concentration (Lockhart and Winzeler, 2000). The degree of mRNA expression is very informative about the state of a cell and the activity of genes; the expression profile of a collection of genes is a major determinant of cellular phenotype and function.

The aims of this section of the project were to:

- Determine (in comparison with urban samples) the acellular *in vitro* bioreactivity of landfill PM_{10} and its sub-fractions ($PM_{2.5-0.1}$ and $PM_{10-2.5}$) using the acellular PSA
- Investigate the effect of antioxidant SELF and metal chelation of PM in the PSA
- Evaluate the effect of PM transition metal-chelation in the PSA
- Perform conventional toxicology and toxicogenomics of EpiAirway™ 3-D tracheobronchial cell model following landfill and urban composite PM_{10} exposure.

6.2 METHODS

6.2.1 MATERIALS AND SOURCES

All sources are UK-based unless otherwise stated.

Material:	Source:
Agarose Tablets 0.5g	Bioline
Ascorbic Acid	Sigma-Aldrich
Biotin-11-UTP	Perkin Elmer
Chloroform	Fisher Scientific
Chelex-100	Sigma-Aldrich
cRNA Cleanup Kit	SuperArray, Frederick, USA
Desferroxiamine Mesylate (DES)	Sigma-Aldrich
DTPA	Sigma-Aldrich
EDTA	Fisher Scientific
EpiAirway™-100	MatTek Corporation, USA

Ethanol	Fisher Scientific
GEArray Expression Analysis Suite	SuperArray, Frederick, USA (now SABiosciences, MD, USA)
Glutathione (Reduced form)	Sigma-Aldrich
Isopropanol (Propan-2-ol)	Fisher Scientific
Molecular Biology Reagent Water (MB H_2O)	Sigma-Aldrich
NanoDrop™ Spectrophotometer	Thermo Scientific
Oligo GEArray® Human Toxicology & Drug Resistance Microarray (OHS-401)	SuperArray, Frederick, USA (now SABiosciences, MD, USA)
Orange/ blue Loading Dye 6x	Promega
Phosphate Buffered Saline (Ca/Mg)	Sigma- Aldrich
QIAshredder	Qiagen
QIAzol Lysis Reagent	Qiagen
RNAlater®	Sigma-Aldrich
RNeasy Mini Kit	Qiagen
Rotisserie Hybridisation Oven	Ultraviolet Products
SDS Solution (20%)	National Diagnostics
SSC Solution (20x)	National Diagnostics
Significance Analysis of Microarrays (SAM)	Stanford University,CA, USA
Tris-Borate-EDTA (10x).	Sigma-Aldrich
Uric Acid	Sigma- Aldrich
UVP Biospectrum Imaging System	Ultraviolet Products

6.2.2 *Plasmid Scission Assay*

The PSA method and materials were described in Chapter 2, section 2.2.4. The $PM_{2.5\text{-}0.1}$ and $PM_{10\text{-}2.5}$ were processed as (i) whole PM suspensions (ii) soluble fractions and (iii) insoluble fractions. See Section 5.2.2 for preparation of concentrated stock solutions. The in/soluble fractions were obtained from whole PM suspensions using the following procedure (Table 6.1).

6.2.2.1 *Surrogate Epithelial Lining Fluid and Metal Chelators*

SELF (working solution; Table 6.2) was prepared from Uric Acid (UA), Ascorbic Acid (AA) and reduced Glutathione (GSH), to a final concentration of 200µM.

SOLUBLE AND INSOLUBLE PM SAMPLES FOR PSA	DURATION
Concentrated PM stock diluted to 1mg/ml in MB H_2O (non-stick eppendorfs)	
Sample agitated on orbital shaker (medium speed)	1h
Sample centrifuged (20,000 x *g*)	1h
Supernatant carefully transferred to fresh eppendorf, -80°C storage (soluble fraction) until use in PSA	
PM pellet resuspended in MB H_2O to original volume, -80°C storage (insoluble fraction) until use in PSA	

Table 6.1: *Preparation of $PM_{2.5-0.1}$ and $PM_{10-2.5}$ soluble and insoluble fractions for use in the PSA.*

SURROGATE EPITHELIAL LINING FLUID
Dissolved uric acid by heating (90°C) in chelex-treated MB H_2O
Allowed to cool to 4°C and protect from light before AA was added
Added GSH (reduced form)
Adjusted to pH 7.4 (chelex-treated NaOH)
Aliquoted into 1ml amber eppendorfs, stored at -80°C until use in PSA

Table 6.2: *Preparation of SELF working solution. Final components 200μM each*

Aliquots of 15μl SELF were added to 5μl of the total or soluble PM at a PM concentration which had been shown to produce maximal damage in the PSA (final SELF concentration 150μM). This SELF-PM mixture was allowed to pre-incubate for 4h, before transfer of 19μl into foil-wrapped tubes for immediate use in the PSA.

6.2.2.2 METAL CHELATORS

The EDTA, DTPA (transition and main-group metal chelators) and DES (putative Fe^{3+}-specific chelator), were prepared in MB H_2O. Concentrated stock solutions of EDTA and DTPA (20mM) were dissolved by overnight agitation in

MB H_2O; the now highly acidic (~pH 2) aqueous DTPA was adjusted to pH 7.4 before use. DES was stored as 2mM samples at -20°C. The plasmid was incubated with whole or soluble PM final concentrations which were found to cause maximal damage in preliminary PSA experiments. All chelators were added as spikes; final concentrations were 10mM (EDTA, DTPA) and 1mM (DES).

6.2.2.3 PSA STATISTICAL ANALYSIS

Data were assessed for normality with the Anderson-Darling test for homogeneity in Minitab 15 (Microsoft Inc., WA, USA). Data were compared for statistical significance between PM sizes (intra-site) and between corresponding PM fractions (inter-site; SPSS 14.0, SPSS Inc. IL, USA); two-way ANOVA and least significant difference *post-hoc* testing. Standard deviation was used to represent experimental variation. Significance was accepted at $p \leq 0.05$, whilst $p \leq 0.01$ indicated high statistical significance. Dose-response curves for the PSA were plotted in MS Office Excel and TD_{50} values (PM concentration required to cause 50% total plasmid DNA damage) were obtained from linear or non-linear regression sigmoidal modelling using GraphPad Prism 2 (GraphPad Software Inc., CA, USA).

6.2.3 EPIAIRWAY™ TISSUE CULTURE AND EXPOSURE

The EpiAirway™ model was purchased from MatTek Corporation as a fully-differentiated tissue, approximately 10mm in diameter and may range from 60 - 110µm thick (Chemuturi *et al.*, 2005; Sexton, K., personal communication, 2008). These were commercially cultured over a period of 3-4 weeks as individual tissue culture inserts, in order to develop the multi-layered structure (Figure 6.1). The final (ready-to-use) tissue inserts were transported in a 24-well plate at 4°C and upon receipt, removed from the shipment gel and placed into 0.9ml media in 6-well plates at 37°C, 5% CO_2 for 24h acclimatisation, prior to dosing (Figure 6.2). Three technical replicates per PM sample were obtained.

The $PM_{2.5-0.1}$, $PM_{10-2.5}$ and composite PM_{10} mixtures, in sterile phosphate buffered saline (PBS; 500µg/ml), were prepared from the lyophilised $PM_{2.5-0.1}$

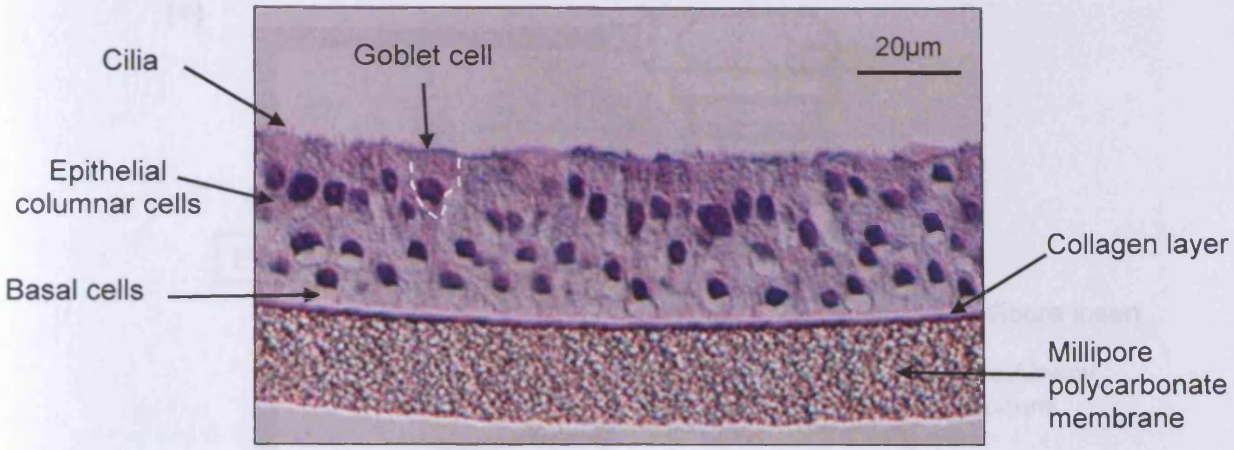

Figure 6.1: *Cross-section of an EpiAirway™ insert. The basal, epithelial and goblet cells (dotted outline) form a 3-D model of the normal human tracheobronchial region (Image courtesy of MatTek Corporation).*

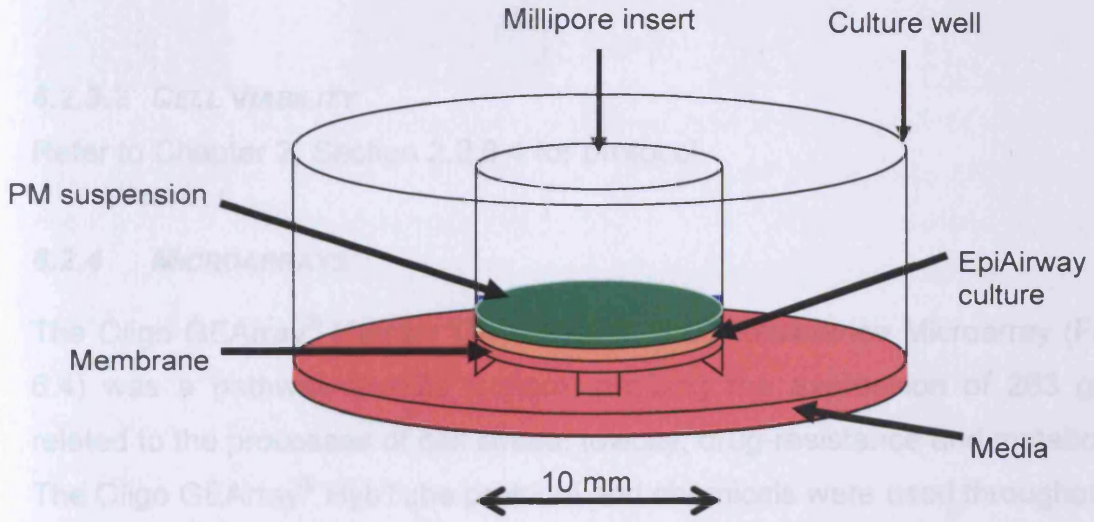

Figure 6.2: *EpiAirway™ culture insert and sample application. The cells were maintained in an air-liquid-interface on a raised semi-permeable platform.*

and $PM_{10-2.5}$ for the landfill and urban HVCI collections of 2007. These test solutions were warmed to 37°C before application of 100µl (n=3; 50µg/cm^2) to the apical surface (24h acute exposure). The structural integrity of the pre- and post-dosed EpiAirway™ tissues were evaluated by measuring the trans-epithelial electrical resistance of the multilayered cell culture (TEER), and cell viability (active metabolism) was measured with the MTT assay.

6.2.3.1 *Tissue Integrity*

Refer to Chapter 2, Section 2.2.6.3 for protocol, and Figure 6.3.

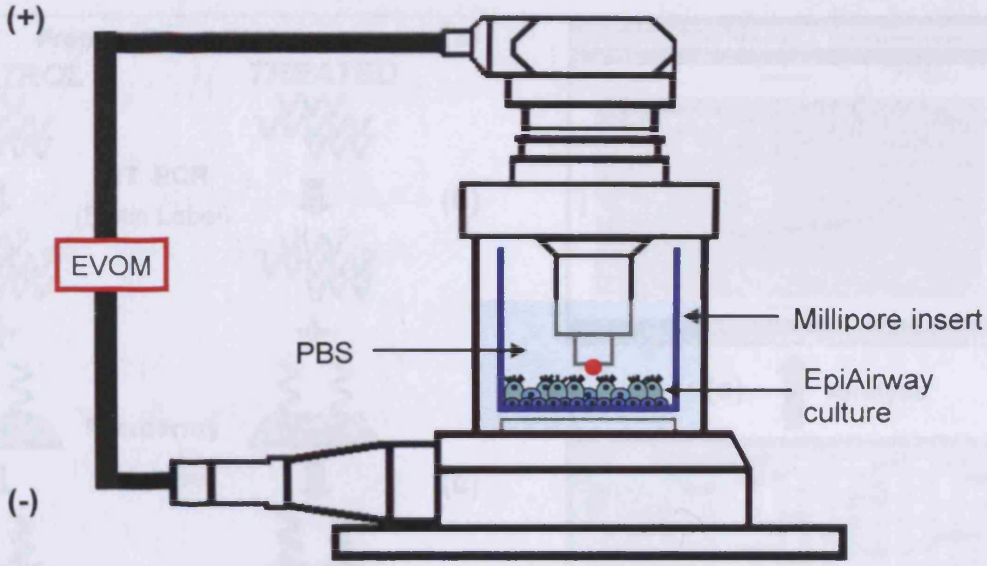

Figure 6.3: *TEER measurement with an Endohm™ chamber linked to Epithelial tissue Volt Ohm Meter (EVOM). Control inserts gave 24h readings of 100-200 Ω/cm².*

6.2.3.2 CELL VIABILITY

Refer to Chapter 2, Section 2.2.6.4 for protocol.

6.2.4 MICROARRAYS

The Oligo GEArray® Human Toxicology & Drug Resistance Microarray (Figure 6.4) was a pathway-specific system, profiling the expression of 263 genes related to the processes of cell stress, toxicity, drug-resistance and metabolism. The Oligo GEArray® HybTube protocol and chemicals were used throughout.

6.2.4.1 RNA EXTRACTION AND PREPARATION

RNA from each EpiAirway™ cell culture was isolated using QIAzol Lysis reagent following the Qiagen protocol (QIAzol Lysis Reagent Handbook 2, 10/2006, Qiagen UK). Detailed below, this is a single step, liquid phase separation procedure, which allows the simultaneous extraction of RNA, DNA and protein.

The cells were harvested by excising the EpiAirway™ platform and scrapping the tissue off the membrane support. The cells were then lysed in QIAzol, followed by centrifugation (12000 x *g*; 30s) in a QIAshredder column, resulting

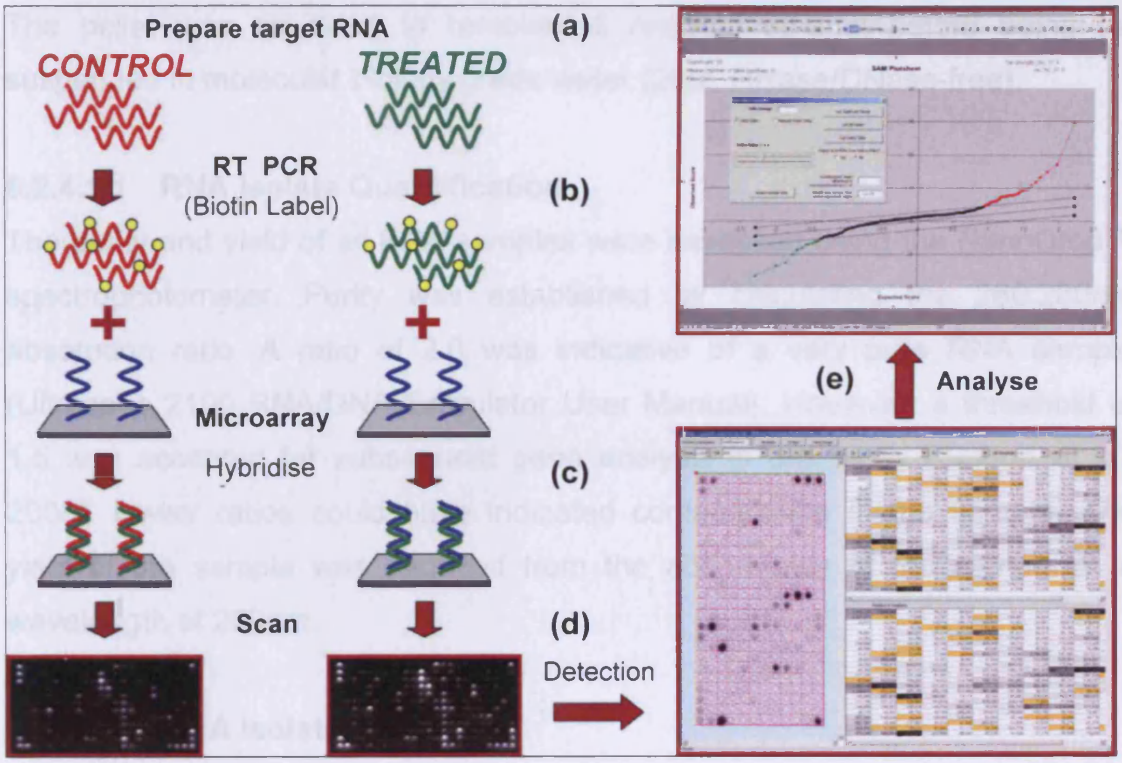

Figure 6.4: *(**a**) RNA was extracted and purified from sample. (**b**) RNA was amplified and labelled by a reverse transcription reaction (**c**) and the labelled cRNA copy was hybridised to the DNA sequences bound to the 2.5 × 3.8cm nylon membrane (array). (**d**) Detection and quantification of array information was achieved using software specific to the type of array used (SABiosciences). (**e**) Global normalisation and statistical analyses were applied (SAM; Stanford University), enabling the relative abundance of each of the gene sequences in two or more biological samples to be compared.*

in a mono-phase solution of guanidine thiocyanate and phenol. All insoluble material and high molecular weight DNA was removed by centrifugation (12000 x *g*; 30s). After the addition of 0.2ml chloroform, the solution was mixed thoroughly, allowed to stand at RTP for 3min and then centrifuged (12000 x *g*, 15min). This resulted in the separation of the solution into three layers: (i) a lower, red organic phase (protein), (ii) a white interphase (DNA) and (iii) upper, colourless aqueous phase (RNA). The aqueous RNA layer was carefully pipetted to a clean eppendorf and 0.5ml isopropanol added. The protein and DNA layers were discarded. After centrifugation (12000 x *g*, 10min), the RNA precipitate formed a pellet. The supernatant was discarded and the pellet was washed in 1ml 75% ethanol and reformed by centrifugation (7500 x *g*, 5min).

The pellet was air dried to remove all residual ethanol before being re-suspended in molecular biology grade water (20µl, RNase/DNase-free).

6.2.4.1.1 RNA Isolate Quantification

The purity and yield of all RNA samples were assessed using the NanoDrop™ spectrophotometer. Purity was established by calculating the 260:280nm absorption ratio. A ratio of 2.0 was indicative of a very pure RNA sample (Ultraspec 2100 RNA/DNA Calculator User Manual). However, a threshold of 1.5 was accepted for subsequent gene analysis in this study (Sexton *et al.*, 2008). Lower ratios could have indicated contamination in the sample. The yield of the sample was deduced from the absorbency of the sample at a wavelength of 260nm.

6.2.4.1.2 RNA Isolate Integrity

An subsample of the RNA was electrophoresed on a 1% agarose gel (2g agarose in 200ml 1x TBE with 4µl of 10mg/ml Ethidium Bromide). Each well was loaded with 200-600ng RNA, 2µl loading dye, and made up to a total of 10µl in MB H_2O. The gel was electrophoresed (90V, 40min) and imaged with UVP Biospectrum Imaging System VisionWorks®.

6.2.4.2 Preparation of Biotin-labelled cRNA Target

The following solutions (Tables 6.3-5) were prepared before proceeding with the scheme presented in Table 6.6 for the generation of a biotin-labelled cRNA target for each sample (EpiAirway™ culture insert) harvested.

Annealing Mix	Volume
RNA	0.5-0.6µg of total RNA
Truelabelling Primer	1µl
MB H_2O	to a final volume of 10µl

Table 6.3: *Preparation of Annealing Mix solution (per sample).*

cDNA Synthesis Master Mix	Volume
RNase-free H_2O	4µl
5 x cDNA Synthesis Buffer	4µl
RNase Inhibitor	1µl
cDNA Synthesis Enzyme Mix	1µl

Table 6.4: *Preparation of cDNA Synthesis Master Mix solution (per sample).*

Amplification Master Mix	Volume
2.5 x RNA Polymerase Buffer	16µl
Biotinylated-UTP, 10mM	2µl
RNA Polymerase Enzyme	2µl

Table 6.5: *Preparation of Amplification Master Mix solution (per sample).*

cRNA Target		Temperature °C	Duration
Prepare Annealing Mix (Table 6.3)			
Incubate		70	10min
Cool to ~ 40°C			
Add cDNA synthesis Master Mix (Table 6.3)			
Incubate (two-stage)	(i)	42	50min
	(ii)	75	5min
Cool to 37°C			
Add Amplification Mix (Table 6.4)			
Incubate		37	16h

Table 6.6: *Preparation of Biotin-labelled cRNA target.*

The generated cRNA reaction mixture contained a large excess of unincorporated nucleotides which would have interfered with the calculation of actual yield of cRNA product required for the array hybridisation. Hence, a cRNA Cleanup Kit (SuperArray, USA) was used for purification. The kit incorporated small-scale chromatography and centrifugation to give a quick and reproducible separation of the probe from contaminants. The probe was eluted

in 10mM Tris Buffer pH 8.0 and stored on ice. NanoDrop quantification of the purified probe was carried out as described in Section 6.2.4.1.1.

6.2.4.3 *Hybridisation of the cRNA Target to the Array*

The microarray membranes were moistened in the supplied hybridisation tubes containing 5ml of deionised water. The water was discarded after 5min and replaced with 2ml pre-warmed (60°C) GEAhyb Hybridisation Solution. The tubes were then placed within standard hybridisation cylinders and secured within the rotisserie hybridisation oven at 60°C for 60min with slow rotation (5 RPM).

Target Hybridisation mix was prepared by adding 2µg of the biotin-labelled cRNA (Section 6.2.4.2) to 0.75ml GEAhyb Hybridisation Solution. The initial Hybridisation Solution from the hybridisation tube was discarded and replaced by this Target Hybridisation mix. This Target (biotin-labelled cRNA) was hybridised onto the microarray membrane overnight at 60°C with slow rotation (5 RPM).

The Target Hybridisation mix was removed from the hybridisation tube and replaced with 5ml Wash Solution 1 (low stringency wash; Table 6.7). The hybridisation tube was placed back in the hybridisation oven with faster agitation (20 RPM, 15min).

Wash Solution 1	Volume
20 x SSC	10ml
20% SDS	5ml
18 MΩ Water	to a final volume of 100ml

Table 6.7: *Preparation of low stringency Wash Solution 1 (2 x SSC, 1% SDS).*

This first wash was discarded and replaced with 5ml Wash Solution 2 (high stringency, Table 6.8) and placed back in the hybridisation oven (20 RPM, 15min). The Wash Solution was removed and the membrane was allowed to cool to room temperature.

WASH SOLUTION 2	VOLUME
20 x SSC	0.5ml
20% SDS	2.5ml
18 MΩ Water	to a final volume of 100ml

Table 6.8: *Preparation of high stringency Wash Solution 2 (0.1 x SSC, 0.5% SDS).*

6.2.4.4 CHEMILUMINESCENT DETECTION

The first step of chemiluminescent detection was to block the unused probe areas on the microarray by the addition of 2ml GEAblocking solution Q to the hybridisation tube, which was incubated for 40min incubation with rotation. The blocking solution was discarded; the next step was to bind the membrane with alkaline phosphatase-conjugated streptavidin (AP-SA) by adding 2ml AP-SA buffer and incubation with gentle rotation (5 RPM, 10min). The AP-SA buffer was then discarded and the membrane was washed several times with Buffers F and G (supplied with array). Finally, 1ml of CDP-Star Chemiluminescent Substrate was added to the hybridisation tube and incubated (5 RPM, 5min). The membrane was removed with forceps and placed into a zip-lock bag for immediate image acquisition.

The chemiluminescence was detected using an UVP Biospectrum Imaging System. This system incorporated a high resolution Hamamatsu 12 bit CCD camera and Visionworks® software. Images were detected using dynamic integration set to take 20 images over a 20min period using a binning sensitivity of 1x1 (maximum sensitivity). The image was then selected on the parameters of maximum number of spots detected without bleeding or overexposure.

6.2.4.5 MICROARRAY STATISTICAL ANALYSIS

The data generated from PBS control and landfill or urban PM_{10}-exposed EpiAirway™ samples were obtained using the GEArray Expression Analysis Software (n=3). An adjusted intensity was taken to be a quantitative measure of gene expression (i.e. spot intensity minus background [membrane] intensity). Preliminary statistical analysis using an Anderson Darling test for normality (p >0.05) on 25 genes (9%) confirmed the raw array data was normally

distributed. In order to compare hybridisation signal intensities across the membranes, a normalised signal intensity for each spot was obtained. Normalisation can be defined as the process of correction for differences in overall array intensity (i.e. background noise). Global normalisation between membranes involved dividing the spot intensity of each gene by the median spot intensity of the whole array to correct for variations between arrays. As the mean value can be distorted by the effects of a few extreme outliers, the median value was considered the most accurate basis for normalisation, thereby reducing the possibility of data variance due to anomalous data points (Balharry *et al.*, 2005).

A null hypothesis of no expression-level difference in individual genes between control and PM_{10} exposure was established. The significance of differentially expressed genes could only result when statistical analyses were applied. Hence, the data was analysed using Significance Analysis of Microarrays method (SAM). SAM correlates gene-expression data to a wide variety of parameters and uses data permutations to provide an estimate of false discovery rates for multiple testing. The resultant statistical value could then be used to determine which genes were significantly, differentially-expressed. Changes in gene-expression were expressed as a ratio (fold-change). The lower cut-off point for identifying relevant genes was set at a ratio of 1.5 (de Vos *et al.*, 2003; Treadwell and Singh, 2004). Genes were also grouped and annotated according to the functional classification allocated by GEArray Expression Analysis Software (Table 6.9).

6.3 RESULTS

6.3.1 PLASMID SCISSION ASSAY

The dose-response curves for all total, soluble and insoluble samples have been provided in Figures 6.5 – 6.7, whilst TD_{50} values for the sufficiently reactive samples are provided in Table 6.10.

GEArray® Human Toxicology & Drug Resistance Microarray
Apoptosis
Cell cycle
Cell growth, Proliferation and Differentiation
Transporters
Response to Stress
Chaperones and Heat Shock Proteins
Transcription Factors and Regulators
Drug Metabolising Enzymes

Table 6.9: *Summary of the main functional gene classes on the Oligo GEArray® Human Toxicology & Drug Resistance Microarray.*

Since no insoluble fractions exhibited a dose-response, these have been omitted from Table 6.10. Although the urban 2007 coarse soluble sample exhibited a linear dose-response, the TD_{50} was found to be greater than 600µg/ml, and classified as a non-reactive sample (data not shown).

6.3.1.1 PM_{10} Collections 2007

The total $PM_{2.5\text{-}0.1}$ fractions at both the landfill and urban sites (Figure 6.5.a) were found to be highly significantly more bioreactive than their corresponding $PM_{10\text{-}2.5}$ and composite PM_{10} samples (Figures 6.5.a and 6.6; $p \leq 0.01$). Landfill 2007 $PM_{2.5\text{-}0.1}$ exhibited a significantly higher ($p \leq 0.05$) oxidative capacity (TD_{50}: 28µg/ml) than the corresponding urban $PM_{2.5\text{-}0.1}$ (TD_{50}: 185µg/ml; Koshy *et al.*, 2009). There was no significant difference between the landfill PM_{10} and $PM_{10\text{-}2.5}$ fractions. The trend of bioreactivity of the landfill 2007 samples was in the order of $PM_{2.5\text{-}0.1}$ >> PM_{10} = $PM_{10\text{-}2.5}$. The trend of bioreactivity of the urban 2007 site ran in the order of $PM_{2.5\text{-}0.1}$ > PM_{10} > $PM_{10\text{-}2.5}$. All the urban 2007 size fractions, excluding $PM_{2.5\text{-}0.1}$, exhibited significantly higher oxidative capacity when compared with their counterparts in the landfill collection ($p \leq 0.05$).

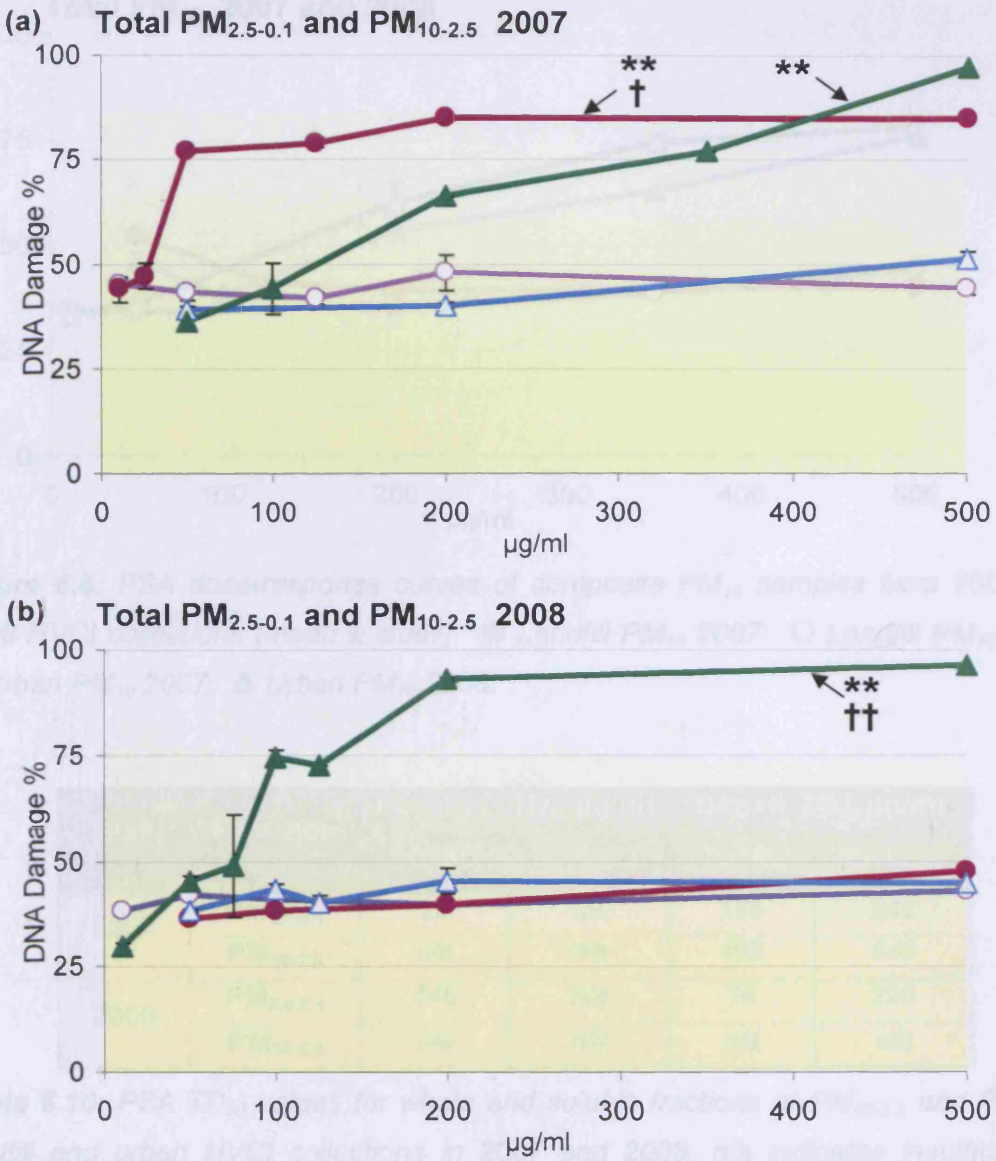

Figure 6.5: *PSA dose-response curves of total $PM_{2.5-0.1}$ and $PM_{10-2.5}$ samples from HVCI collections of* ***(a)*** *2007 and* ***(b)*** *2008 (mean ± stdev).* ● *Landfill $PM_{2.5-0.1}$;* ○ *Landfill $PM_{10-2.5}$;* ▲ *Urban $PM_{2.5-0.1}$;* △ *Urban $PM_{10-2.5}$. Between-size fraction significance* ** *p ≤0.01; Between-site significance* † *p ≤0.05;* †† *p ≤0.01.*

Both landfill and urban $PM_{2.5-0.1}$ soluble fractions caused significant oxidative damage in the PSA (Figure 6.7.a; TD_{50}: 99 and 248µg/ml, respectively). Although the landfill insoluble $PM_{2.5-0.1}$ elicited a dose- response curve which closely mirrored that of the corresponding soluble fraction, it was not sufficient for extrapolation of a TD_{50} value from the limited data available. The urban $PM_{2.5-0.1}$ insoluble sample showed no dose-response. The landfill and urban $PM_{10-2.5}$ soluble and insoluble fractions elicited minimal responses in the PSA,

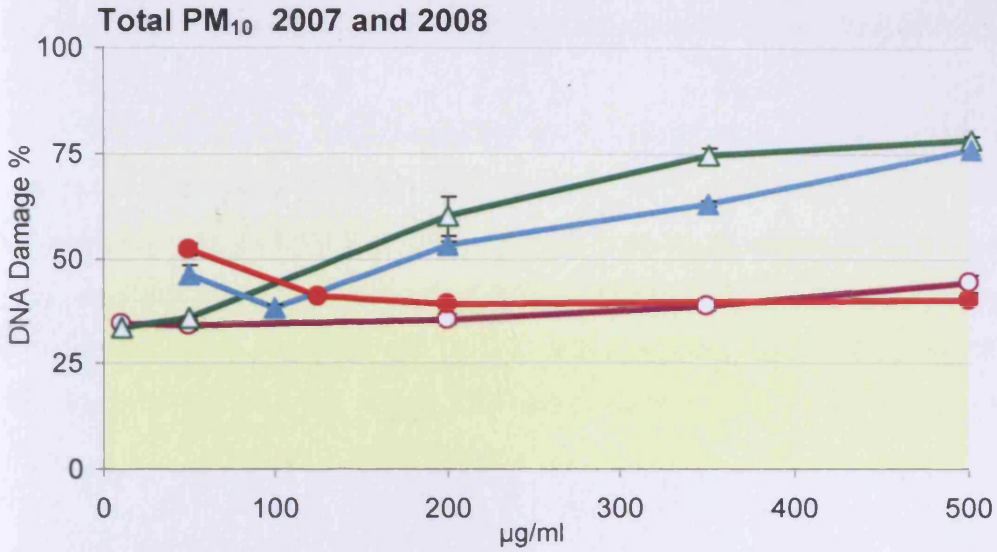

Figure 6.6: *PSA dose-response curves of composite PM_{10} samples from 2007 and 2008 HVCI collections (mean ± stdev).* ● *Landfill PM_{10} 2007;* ○ *Landfill PM_{10} 2008;* ▲ *Urban PM_{10} 2007;* △ *Urban PM_{10} 2008.*

YEAR	FRACTION	LANDFILL TD_{50} µg/ml		URBAN TD_{50} µg/ml	
		TOTAL	SOLUBLE	TOTAL	SOLUBLE
2007	$PM_{2.5-0.1}$	28	99	185	248
	$PM_{10-2.5}$	*n/a*	*n/a*	493	*645*
2008	$PM_{2.5-0.1}$	*548*	*n/a*	76	220
	$PM_{10-2.5}$	*n/a*	*n/a*	*n/a*	*n/a*

Table 6.10: *PSA TD_{50} values for whole and soluble fractions of $PM_{10-2.5}$ and $PM_{2.5-0.1}$ landfill and urban HVCI collections in 2007 and 2008. n/a indicates insufficient or absence of a dose-response.*

but the urban 2007 $PM_{10-2.5}$ soluble sample adhered to a linear regression, (r^2 >0.99), resulting in a TD_{50} of 645µg/ml.

6.3.1.2 PM_{10} COLLECTIONS 2008

Neither landfill total $PM_{2.5-0.1}$ or $PM_{10-2.5}$ samples exhibited a sigmoidal dose-response in the PSA (Figure 6.5.b). However, a linear regression (r^2 >0.99) of the landfill total $PM_{2.5-0.1}$ resulted in a TD_{50} of 548µg/ml. This lack of oxidative activity was also found in the landfill soluble and insoluble preparations, irrespective of PM size fraction (Figure 6.7.c-d). In contrast, the urban $PM_{2.5-0.1}$ total and water-soluble (Figures 6.5.b and 6.7.c) samples were shown to be

highly oxidative, resulting in TD_{50} values of 76µg/ml and 220µg/ml, respectively.

The urban $PM_{10-2.5}$ total, soluble and insoluble samples were all poorly responsive in the PSA. The landfill 2008 composite PM_{10} and $PM_{10-2.5}$ PSA responses were not significantly different from each other. This low bioreactivity was also exhibited by the landfill $PM_{2.5-0.1}$ fraction. The observed bioreactivity of the landfill 2008 samples ran in the order of $PM_{2.5-0.1}$ = PM_{10} = $PM_{10-2.5}$. The bioreactivity trend of the urban 2008 site was in the order of $PM_{2.5-0.1}$ >> PM_{10} > $PM_{10-2.5}$.

6.3.1.3 SELF AND METAL CHELATION

The three most bioreactive total PM samples (landfill and urban 2007 $PM_{2.5-0.1}$, and urban 2008 $PM_{2.5-0.1}$) were selected for further investigation with antioxidants and metal chelators. None of the coarse samples were sufficiently bioreactive to warrant further investigation.

The metal chelators EDTA, DTPA and DES were the most ameliorating upon the $PM_{2.5-0.1}$ oxidant activity ($p \leq 0.01$). SELF had comparatively the least, albeit highly significant ($p \leq 0.01$) protective effect, upon the plasmid DNA (Figure 6.8).

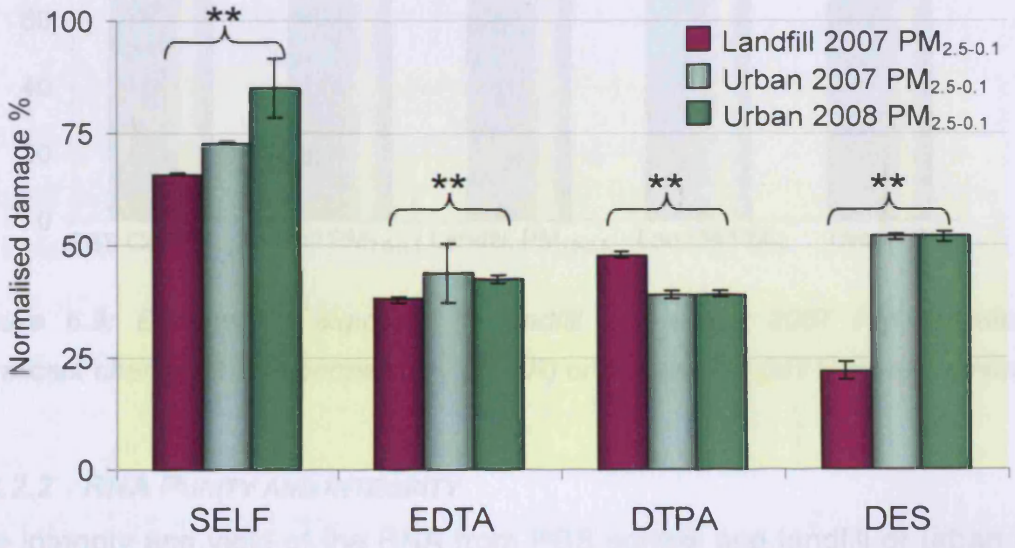

Figure 6.8: *Effect of co-incubation of total $PM_{2.5-0.1}$ (sufficient to cause maximal damage) with SELF (150µM) or metal chelators (EDTA, DTPA: 10mM; DES: 1mM) upon plasmid DNA damage. All data were normalised to untreated $PM_{2.5-0.1}$ and deemed highly significantly protective (** $p \leq 0.01$) compared to control inserts.*

The order of damage attenuation in the landfill $PM_{2.5-0.1}$ sample was DES > EDTA > DTPA > SELF. The order of attenuation in both urban (2007 and 2008) $PM_{2.5-0.1}$ samples were EDTA = DTPA > DES > SELF.

6.3.2 *EpiAirway™ Exposure*

6.3.2.1 Tissue integrity and Cell Viability

Inserts were assessed for tissue integrity pre- and post-PM exposure. No significantly different TEER values were obtained from tissues exposed to the PM_{10} when compared to PBS negative control (Figure 6.9). Post-exposure mitochondrial activity, determined by the MTT assay, did not reveal a statistically significant difference in cell viability in PM_{10}-exposed tissue. Although the MTT reading of the landfill composite PM_{10} was elevated in comparison to the urban composite PM_{10}, this was not significant ($p > 0.05$).

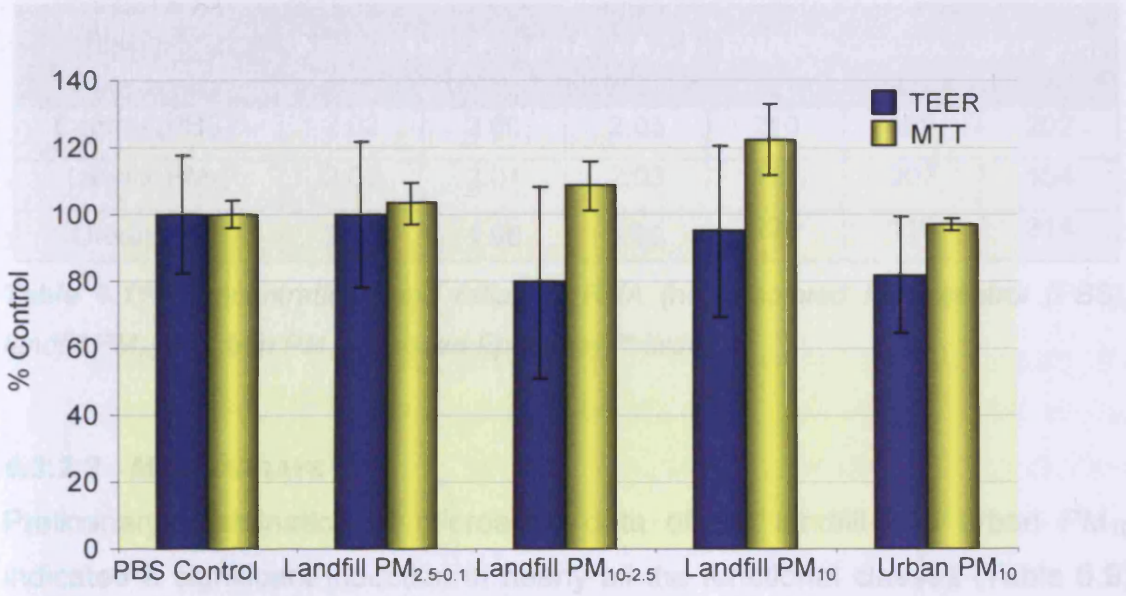

Figure 6.9: *EpiAirway™ exposure to landfill and urban 2007 PM revealed no significant changes in cell permeability (TEER) or cell viability (MTT) measurements.*

6.3.2.2 RNA Purity and Integrity

The integrity and yield of the RNA from PBS control and landfill or urban PM_{10} exposed EpiAirway™ (n=3) is presented in Table 6.11. The results indicated that each sample was within the desired ratio of 1.8 – 2.0, with an acceptable yield of RNA mass. Isolated RNA was electrophoresed on a 1% agarose gel (Figure 6.10) to check RNA integrity and for the presence of unwanted genomic

contamination. There was minimal genomic DNA contamination and the observed bands were free from smears (which would have been an indication of RNA degradation).

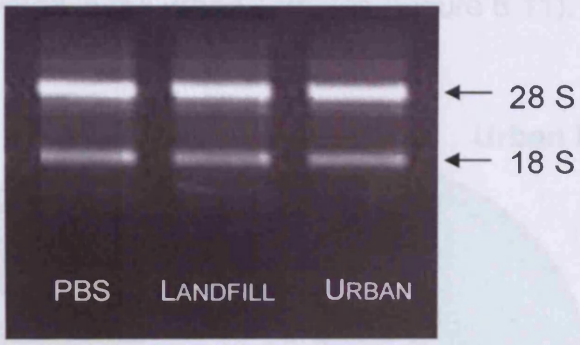

Figure 6.10: *The 28S:18S subunit ratio of rRNA indicated the integrity of the sample RNA; a ratio greater than 1.5 was considered to be an indicator of good quality.*

EXPOSURE OF EPIAIRWAY™	ABSORBANCE RATIO (A_{260}/A_{280})			RNA CONCENTRATION (ng/µl)		
Control (PBS)[a]	2.02	2.00	2.05	210	169	202
Landfill PM_{10}	2.03	2.01	2.03	171	207	154
Urban PM_{10}	2.00	1.96	1.99	229	116	314

Table 6.11: *Concentrations and ratios of RNA (n=3) isolated from control (PBS), landfill PM_{10} or urban PM_{10}-exposed EpiAirway™ tissue.*

6.3.2.3 MICROARRAYS

Preliminary examination of microarray data of the landfill and urban PM_{10} indicated a significant induction in nearly all the functional classes (Table 6.9) represented by the microarray. Landfill PM_{10} caused an up-regulation in 66 genes, whilst urban PM_{10} treatment resulted in 57 up-regulated genes (fold-change >1.5-fold; Appendix C; Koshy *et al.*, 2009). At this stage, the two major groups were the (i) drug metabolism and (ii) chaperones and heat shock proteins, together accounting for over 50% of all genes altered more than 1.5-fold by either landfill or urban PM_{10}. No drug transporter-associated genes were significantly altered in either landfill or urban PM_{10} exposed tissue in comparison to the PBS (control).

Further filtering of this data set resulted in 21 genes from either landfill PM_{10} or urban PM_{10} exposure which were found to be up-regulated over 3-fold in comparison to PBS controls (Table 6.12). This identified 5 genes which were equally dominant in both landfill and urban samples; 9 were elevated in the landfill, and 7 were elevated in the urban samples (Figure 6.11).

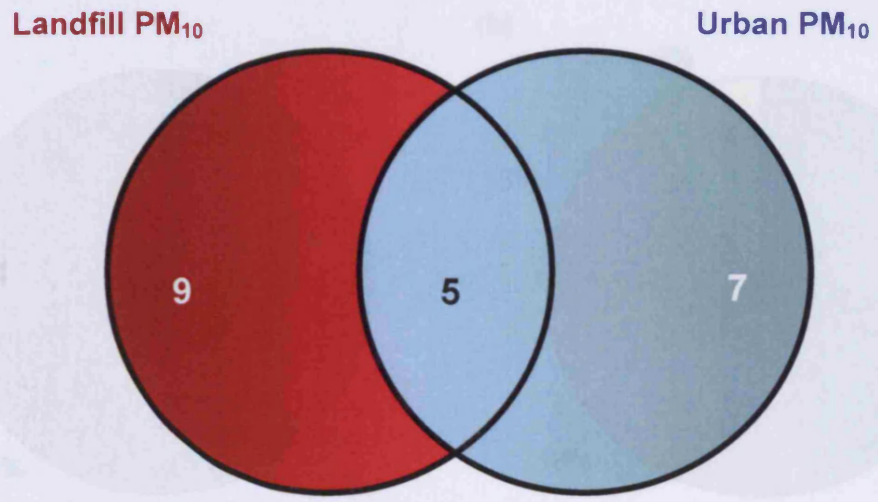

Figure 6.11: *Venn diagram illustrating the overlap of up-regulated gene expression between landfill and urban PM_{10}-exposed EpiAirway™. Fold change 3-fold greater than PBS controls.*

The distribution of induced genes, arranged into functional groups by percentage, showed that although both landfill and urban PM_{10} elicited similar responses in the EpiAirway™ system, the drug metabolism and apoptosis groups exhibited wider up-regulation from urban PM_{10} treatment (Figure 6.12).

Of the eight functional groups represented by the microarray (Table 6.9), five of these groups were elevated by 3-fold in both landfill and urban PM_{10} treatments (Table 6.12). These genes were: *NADPH dehydrogenase quinone 1*, *Peroxiredoxin 1*, *Macrophage Migration Inhibitory Factor*, *Heat Shock Protein 90kDa α (class A member 2)*, and *DNA-damage Inducible Transcript 3* (also known as *GADD153*). No apoptosis-related genes were induced by landfill PM_{10}, but there was greater than 3-fold induction of the pro-apoptotic gene *TRADD* by urban PM_{10}. The major gene family induced in this data subset for

landfill PM_{10} were chaperones and heat shock proteins, whilst urban PM_{10} treatment resulted in preferential expression of the drug metabolism group.

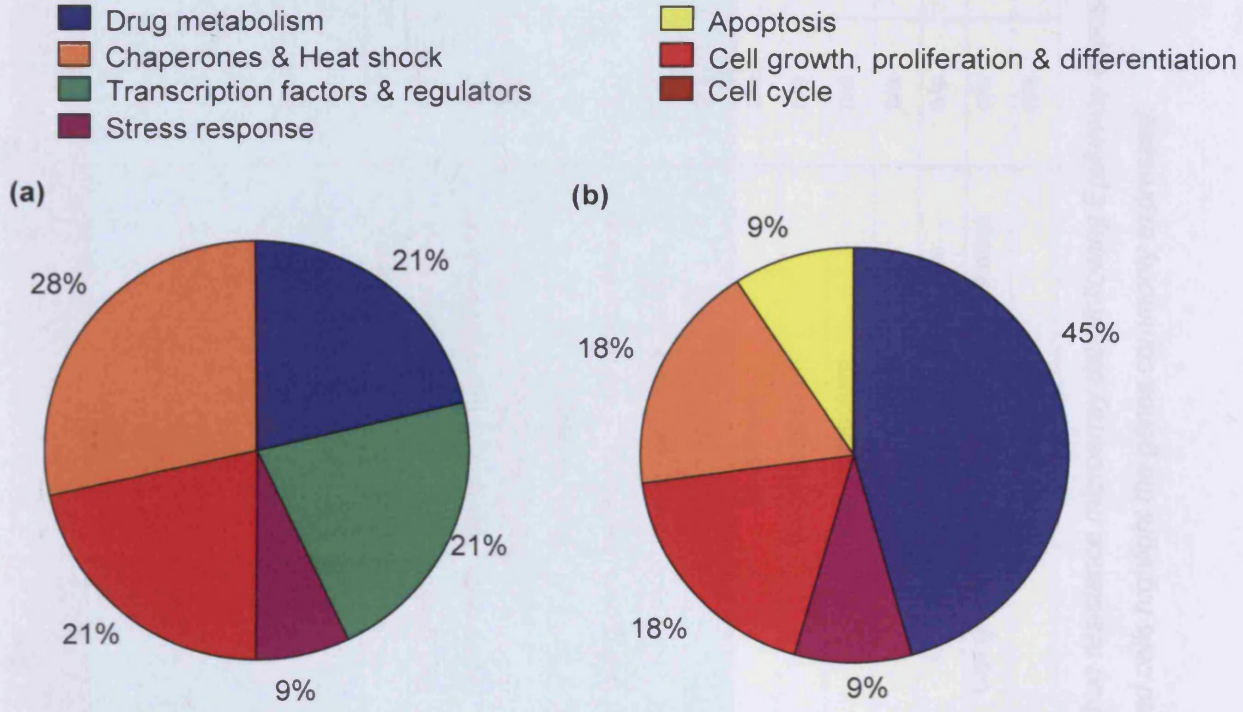

Figure 6.12: *Distribution of altered genes in EpiAirway™ tissue, grouped into functional categories by percentage (up-regulated ≥3 fold, p ≤0.05).* ***(a)*** *Landfill PM_{10} = 14 genes;* ***(b)*** *urban PM_{10} = 12 genes.*

6.4 DISCUSSION

6.4.1 PLASMID SCISSION ASSAY

The oxidative capacity of inhaled PM_{10} has been proposed as a central hypothesis of PM_{10}-mediated adverse health effects (González-Flecha, 2004; Schwarze *et al.*, 2006). The PSA has proven to be a valuable tool in the primary investigation of bioreactivity for a range of environmental studies (Andrewes *et al.*, 2004; Risom *et al.*, 2005; Koshy *et al.*, 2007). In this context, it was utilised to gauge the bioreactivity of landfill $PM_{2.5\text{-}0.1}$, $PM_{10\text{-}2.5}$, and composite PM_{10} in comparison with urban samples.

GENE ID	GENE NAME	GENE FAMILY	FOLD CHANGE (+)	
			LANDFILL	URBAN
CYB5R3	Cytochrome b5 reductase 3	Drug metabolism	3.4	n/a
RELB	v-rel reticuloendotheliosis viral oncogene homolog B	Transcription factor	3.6	n/a
NR1I3	Nuclear receptor subfamily 1 group 1 member 3	Transcription factor	3.4	n/a
NFKBIA	Nuclear factor of kappa light polypeptide	Transcription factor	4.8	n/a
CCT8	Chaperonin containing TCP1, subunit 1	Chaperones and heat shock proteins	3.3	n/a
HYOU1	Hypoxia up-regulated 1	Chaperones and heat shock proteins	3.3	n/a
HSPB1	Heat shock 27kDa protein	Chaperones and heat shock proteins	3.1	n/a
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Cell growth, proliferation and differentiation	3.2	n/a
CDKN1A	Cyclin-dependent kinase inhibitor 1A	Cell growth, proliferation and differentiation	4.4	n/a
NQO1	NADPH dehydrogenase, quinone 1	Drug metabolism	4.5	4.1
PRDX1	Peroxiredoxin 1	Drug metabolism	4.3	3.7
MIF	Macrophage migration inhibitory factor	Cell growth, proliferation and differentiation	3.0	3.2
HSP90AA2	Heat Shock Protein 90kDa α (cytosolic) class A member 2	Chaperones and heat shock proteins	3.1	3.0
DDIT3	DNA-damage inducible transcript 3	Stress response	3.3	3.2
TRADD	TNFRSF1A-associated via death domain	Apoptosis	n/a	3.1
NAT2	N-acetyl transferase 2	Drug metabolism	n/a	3.6
SOD2	Superoxide dismutase 2	Drug metabolism	n/a	3.8
MGST3	Microsomal glutathione S-transferase 3	Drug metabolism	n/a	3.3
HSP90AB1	Heat Shock Protein 90kDa α (cytosolic) class B member 1	Chaperones and heat shock proteins	n/a	3.1
CDK4	Cyclin-dependent kinase 4	Cell growth, proliferation and differentiation	n/a	3.3
NUDT1	Nudix (nucleoside diphosphate linked moiety) type motif 1	Stress response	n/a	5.1

Table 6.12: *Differential gene expression obtained from toxicology and drug resistance microarray data following EpiAirway exposure to composite landfill or urban PM_{10} (21 up-regulated genes). Green coloured cells highlight the genes commonly expressed.*

The composite PM_{10} sample was a representation by mass, of the fine and coarse fractions; hence it did not take particle number into account and resulted in the weight of the final PM_{10} being preferentially influenced by the $PM_{10-2.5}$ fraction, which provided the majority of particle mass, but minority of particle numbers. The smallest sized PM (<2μm), which were a minor contribution to PM_{10} mass, have been shown to penetrate distal airways, reaching the alveolar regions, and exerting greater toxicity than coarse PM (Lippmann *et al.*, 2000; Donaldson *et al.*, 2005; Anderson *et al.*, 2006). As several recent studies have presented strong evidence supporting particle number as a more effective gauge of toxicity and adverse health effects, the relevance of particle size to toxicity must not be over-looked in favour of particle mass (Lighty *et al.*, 2000; Oberdörster, 2001; Englert, 2004). Nonetheless, the potential toxicity of the larger fraction of PM_{10} must still be investigated, as it comes into contact with the airways (Browne *et al.*, 1999; Salvi and Holgate, 1999; Valavanidis *et al.*, 2008).

The greatest bioreactivity was observed from both the landfill and urban $PM_{2.5-0.1}$ samples in 2007, yet the following year in 2008, only the urban $PM_{2.5-0.1}$ sample exhibited significant plasmid DNA damage. This high bioreactivity was preserved in the soluble (bioavailable) fraction and absent from the insoluble (biopersistent) residues. This was an interesting finding, as the low TD_{50} values also corresponded to the high metal solubility of these three samples (see Section 5.3.2.3), supporting the role of metals in the plasmid DNA damage. Several studies have shown increased respiratory and cardiovascular adverse effects are linked to the soluble components of PM (McNeilly *et al.*, 2005; Huang and Ghio, 2006). Adamson *et al.*, (1999) observed *in vivo* adverse pulmonary effects from the metal-rich soluble fraction of the urban particulate, EHC-93. Similarly, increased airway reactivity and elevated signs of inflammation have also been linked to the PM metal concentrations (Pritchard *et al.*, 1996). The TD_{50} values obtained from the three most reactive samples were comparable to PM_{10} bioreactivity of Beijing indoor smokers' homes (100μg/ml: Longyi *et al.*, 2005), south Wales industrial (66 to 175μg/ml: Moreno *et al.*, 2004a) and London 1958 black smoke (163μg/ml: Whittaker, 2003). Samples from which a dose-response curve (and hence TD_{50}) could not be obtained, were deemed non-reactive in the PSA.

The metal-driven oxidative capacity of the samples that were significantly reactive in the PSA was confirmed by the inclusion of antioxidants and metal chelators. The greatest amelioration of the landfill 2007 $PM_{2.5-0.1}$ was seen by inclusion of DES, whilst EDTA and DTPA yielded equal levels of protection. The chelator concentrations (10mM of EDTA and DTPA; 2mM of DES) were established following PSA method optimisation, and were sufficiently in excess of the transition metal concentrations to avoid any scenarios of pro-oxidant action by the chelators (Balcerczyk *et al.*, 2007). DES is known to have high affinity for Fe, as well as Cu, Ni and Co; EDTA and DTPA are excellent transition and main-group metal chelators, hence they can sequester Fe, Cu, Ni, Zn, V, Cr (Mudway *et al.*, 2005; Soylak and Tuzen, 2006). Since Ni was found to be poorly soluble in the chelated PM samples, it was unlikely to be a contributor to the oxidative activity. There have been many conflicting reports on whether the addition of EDTA and DTPA confers antioxidant or pro-oxidant properties. The pro-oxidant nature of EDTA or DTPA may be explained by the ability of these multidentate ligands to bind Fe^{3+} in a conformation that allows participation in redox reactions (Engelmann *et al.*, 2003). In the case of the PM-chelator incubations, the chelator concentrations used were hugely in excess, theoretically preventing pro-oxidant activity in the treatment. The finding that DES provided the most effective protection against landfill $PM_{2.5-0.1}$-inflicted plasmid damage was surprising, since both DTPA and EDTA target a much wider range of metals than DES. The preferential antioxidant activity of DES awaits further investigation.

The oxidative bioreactivity of the bioavailable (soluble) PM fractions used in this investigation correlated with the observed redox metal content (Chapter 5). As these metals (Fe, V, Cu, Co, Cr, Zn and Pb) were concentrated in the smaller size fraction, forming a larger proportion of the total PM mass in $PM_{2.5-0.1}$, it was unsurprising to see the greatest PSA bioreactivity from this fraction. These metals were preferentially fractionated into the soluble fraction, which inflicted similar levels of oxidative damage in the PSA. These results were also in agreement with other studies that report smaller sizes of PM, and their soluble portions, as being more reactive *in vitro* (Salonen *et al.*, 2000; Diociaiuti *et al.*, 2001; Risom *et al.*, 2005) and *in vivo* (Tao *et al.*, 2003). Iron has been heavily implicated in both *in vitro* and *in vivo* oxidative stress, most likely via Fenton

chemistry (Halliwell *et al.*, 1987, 2009; Dusseldorp *et al.*, 1995). Copper can also participate in Fenton chemistry and has been shown to exacerbate ROS-mediated cellular effects (Kennedy *et al.*, 1998; Wilson *et al.*, 2002). Vanadium has been shown to be highly oxidative in other PM toxicity studies (Dye *et al.*, 1999; Ghio, 2002) and might also have been a contributor to the bioreactivity of the three samples of interest. Although not a redox metal *per se*, PM-associated Zn has been linked to genotoxicity (Valko *et al.*, 2005) and adverse cardiovascular and pulmonary effects (Prieditis and Adamson, 2002; Kodavanti *et al.*, 2008). In addition to ROS-mediated plasmid DNA damage, metals such as Zn react directly with the negatively charged phosphodiester backbone of the DNA molecule, causing shearing. Iron is also known to have high affinity for DNA, thereby lending itself to continuous generation of free radicals in close proximity to the DNA (Kasprzak, 2002). Similarly, Pb has been reported to interact with DNA in mechanisms which are not yet fully understood, but are believed to result in single and double DNA strand breaks (Woźniak and Blasiak, 2003).

As the respiratory tract lining fluid is critical in the physical and biochemical defence in the lung against exogenous pollutants, it was decided to use a validated replica (SELF) to modulate the bioreactivity of samples in the PSA, thereby confirming the PM capacity to generate oxidative stress *in vitro* (Zielinski *et al.*, 1999; Mudway *et al.*, 2004). The SELF mixture used in the PSA was a simplistic replica of the much more complex respiratory tract lining fluid; nonetheless, it did reveal interesting results. The PM pre-treated with SELF caused significantly less damage than untreated PM in the PSA; this attenuation was not to the same extent as seen with the PM-chelator treatments. One reason for this was postulated to be the pro-oxidant effect of certain components within the SELF. Ascorbic acid has been shown to be susceptible to auto-oxidation in cell culture media, possibly generating $^{\bullet}OH$ radicals in the presence of a transition metal such as Fe^{3+} which is reduced to Fe^{2+} by AA, and hence, becomes available for Fenton chemistry (Pan *et al.*, 2004). Ascorbic acid is also known to mobilise iron from particulate matter (Tao *et al.*, 2003; Duarte *et al.*, 2007). Nonetheless, the reduced bioreactivity was in agreement with data reported by previous investigators (Greenwell *et al.*, 2002a). Inclusion of metal chelators such as EDTA or DES may be a prudent

addition in future investigations, as this may help minimise AA auto-oxidation, however, since this may also lead to interpretative difficulties, a dose-response investigation would need to be performed.

Since the vast majority of urban $PM_{2.5}$ is believed to comprise ultrafine particles from combustion processes (BéruBé *et al.*, 2007), it seems plausible, based on the high metal solubility of these samples (Chapter 5), to suggest a large contribution of DEP to this sample. As well as redox active transition metals, DEP are also known to contain redox active organic compounds, including quinones and PAHs (which themselves can give rise to quinones). These organic compounds may be adsorbed onto the surface of PM. Future work with the PSA should aim to validate its use solely for quinone assessment. In addition, as this study made no attempt to control the pH of the PM samples, future work should aim to allay concerns over pH effects in the PSA.

Overall, the landfill composite PM_{10} bioreactivity was dominated by the less reactive $PM_{10\text{-}2.5}$ fraction, whilst the urban composite PM_{10} bioreactivity was dictated by the more oxidant $PM_{2.5\text{-}0.1}$ fraction. There was an above-basal level of damage caused in the PSA, at increasing concentrations approaching 1000µg/ml of $PM_{10\text{-}2.5}$ samples which were deemed to be non-responsive in the PSA (data not shown). This may have been due to a mechanical particle effect upon the DNA molecular structure. It was an interesting finding, as this suggested the biopersistent fractions of inhaled PM_{10} may yet cause chronic low levels of damage via mechanical interference (Diociaiuti *et al.*, 2001).

6.4.2 EpiAirway™ Exposure

As there were no significant adverse effects detected by TEER measurements, nor by the MTT viability assay upon exposure to any of the three landfill PM fractions, or from the composite urban PM_{10}, it was concluded that the EpiAirway™ tissue did not exhibit cytotoxic effects. The TEER values of control EpiAirway™ inserts were in range of manufacturer (MatTek Corp.) literature, which stated 475-675Ω/cm^2 as indicative of tight-junction integrity, whilst other investigators have reported control EpiAirway™ TEER values of approximately 180-210 Ω/cm^2 (Chemuturi *et al.*, 2005; Sexton, 2008). A search of the

literature provided no evidence of the use of microarrays in the investigation of landfill PM_{10}. A handful of toxicogenomics studies have been published. These often involved laboratory animal lung or *in vitro* cell exposures (Kooter *et al.*, 2005; Wang *et al.*, 2007; Thomson *et al.*, 2009), human cell lines such as. bronchial epithelial BEAS-2B, macrophage THP-1 (Watterson *et al.*, 2007) and human primary airway cells (nasal epithelium: Rumelhard *et al.*, 2007). Overall, these studies support the pathological evidence of inflammation and oxidative stress. However, experimental differences, such as particle type, dose and exposure duration mean these data may be considered as part of a wider database.

6.4.2.1 Stress Response, Chaperones and Heat Shock proteins

Interestingly, there were some significant changes detected by the microarray experiments, and the absence of down-regulated genes following either landfill or urban PM_{10}-exposure was notable. The significant up-regulation of several members of the chaperones and heat shock proteins group following acute exposure to landfill PM_{10} may be indicative of a cellular stress response. Of note, the significant up-regulation of a number of endoplasmic-reticulum (ER) associated genes might be an indication of a disruption to ER homeostasis, which is the term given to the balance between ER function and ER capacity. As the ER is an important site for synthesis and assembly of proteins, its impaired function (ER stress) could result in unfolded or misfolded proteins. Up-regulation of *HYOU1* (also known as *HSP70-12A*), occurs at the initiation of the ER stress response in order to assist with protein folding, when the ER protein-folding machinery is over-whelmed. If the increase in ER capacity is insufficient, apoptosis may ensue (Oyadomari and Mori, 2004); hence *HYOU1* may have had a cytoprotective role. PM_{10} has been shown to up-regulate *HSP70* in the A549 epithelial cell line, and this effect was prevented by administration of antioxidants (Ramage and Guy, 2004). Another indication of ER stress was the elevated levels of *DDIT3*, a pro-apoptotic gene via disruption of Ca^{2+} homeostasis (Lindenmeyer *et al.*, 2008). *DDIT3* is a ubiquitously expressed transcription factor, and although named as *DNA-damage Inducible Transcript 3*, it is believed to be more responsive to ER stress, rather than DNA damage *per se* (Oyadomari and Mori, 2004). The up-regulation of other genes belonging to the chaperones functional group: *CCT8* (actin and tubulin folding), *HSPB1*

(organisation of actin during environmental stress response) and *HSP90AA2* (folding of some cytosolic cell signalling molecules, e.g. tyrosine kinases) also indicated increased activity of ER in landfill PM_{10}-exposed tissue.

Following urban PM_{10} exposure, the levels of *HSP90AA2, HSP90AB1* and *DDIT3* genes were significantly elevated. This indicated a possibly similar regulation of cellular cytoskeleton. Exposure to $PM_{2.5}$ has been shown to cause perturbations of actin and tubulin in lung epithelial cells (Calcabrini, 2004) and cytoskeletal remodelling (Verheyen *et al.*, 2004). This could have been a physical response of the epithelium to the presence of particulate matter at the apical surface. Interestingly, the up-regulation of *NUDT1* by urban PM_{10} may have had anti-mutagenic effects, as this gene codes for a protein that hydrolyses aberrantly oxidised purine nucleotides, such as the 8-oxodGTP moiety, thereby preventing their misincorporation into DNA and avoiding base-pair transversions (Bräuner *et al.*, 2007). This was highly pertinent to the hypothesis that ROS plays a key role in cellular damage, inflammation and carcinogenesis following exposure to PM (Ichinose *et al.*, 1997; Møller *et al.*, 2008).

6.4.2.2 Drug Metabolism

Following landfill PM_{10} exposure, the drug metabolism genes, *PRDX1*, *NQO1* (anti-oxidant functions) and *CYB5R3* (xenobiotic metabolism) were up-regulated by landfill PM exposure, with *PRDX1* being concomitantly elevated in the urban PM_{10} exposed tissue. *PRDX1* is a member of the peroxiredoxin family, which reduces cellular H_2O_2 to H_2O, thereby suppressing ROS propagation (Sue *et al.*, 2005). The up-regulation of *NQO1* would lead to the conversion of PM-associated quinones to hydroquinones, thus attenuating the cytotoxicity of quinone free radicals and redox cycling (Lewis *et al.*, 2001). The involvement of organic components have been shown to mediate inflammation, hence the up-regulation of *NQO1* may have a cytoprotective function (Baeza-Squiban *et al.*, 1999). As quinones can arise from combustion-derived material in PM_{10}, the up-regulation in *NQO1* may hold biological plausibility and has also been reported at elevated levels in immortalised human bronchial cells which were exposed to $PM_{2.5}$ (Baulig *et al.*, 2009).

Following urban PM_{10} exposure, the gene changes in EpiAirway™ tissue were strongly indicative of a response to ROS and reactive nitrogen species (RNS). The antioxidant enzyme products of *NQO1, PRDX1, NAT2, SOD2* (also known as Mitochondrial SOD) and *MGST3* are involved with several biotransformation and conjugation reactions. *SOD2* is the mitochondrial isoform of the three variants of SOD which are known to exist in humans (the other two being extracellular or cytosolic), and converts $O_2^{\bullet-}$ to H_2O_2 and O_2; it has been shown to repress $PM_{2.5}$-mediated DNA damage *in vitro* (Dellinger *et al.*, 2001). The *NAT2* and *MGST3* products are phase II intracellular xenobiotic metabolising enzymes that detoxify RNS and ROS by conjugation with acetyl CoA and GSH reductants (Hayes *et al.*, 2005; Valko *et al.*, 2007). As the combustion-derived urban $PM_{2.5}$ was proven to be capable of generating ROS in the PSA, and had greater representation in the composite urban PM_{10} sample, this oxidative-stress response was supported by the previous physicochemical characterisation (Chapters 4 and 5).

6.4.2.3 TRANSCRIPTION FACTORS

Following landfill PM_{10} exposure, the up-regulation of the transcription factor *NR1I3* may have lead to increased production of xenobiotic metabolism enzymes, including members of the cytochrome P450 family, which could in turn, have a cytoprotective effect (Pascussi *et al.*, 2003). The *CYB5R3* product in epithelial tissue is the enzyme responsible for reducing Cytochrome b5 in mitochondria, and is also involved with CYP450 activity. The *RELB* and *NFKBIA* genes both play regulatory roles in NFκB activation. The NFκB pathway has been shown to be activated by ROS and plays a central role in regulating expression of many genes involved in the inflammatory and immune response in human airway epithelium (Baeza-Squiban *et al.*, 1999; McNeilly *et al.*, 2005). However, the simultaneous up-regulation of *RELB* and *NFKBIA* appeared to be counter-intuitive as the elevated *RELB* may have led to up-regulation of the NFκB signalling pathway, whilst *NFKBIA* pointed to suppression of active NFκB (May and Ghosh, 1999).

6.4.2.4 CELL GROWTH, PROLIFERATION AND DIFFERENTIATION

Following landfill PM_{10} exposure, the up-regulation of two members of the cyclin-dependent kinase inhibitors, *CDKN2A* (a well-known tumour suppressor

gene) and *CDKN1A* suggested induction of cell cycle arrest and an anti-mutagenic status (Kanellou *et al.*, 2009; Su *et al.*, 2009). In contrast, the exposure of urban PM_{10} resulted in up-regulation of *CDK4*; inferring a promotion of the cell cycle that could have had a pro-mutagenic role.

Both landfill and urban PM_{10} exposures caused an up-regulation of the pro-inflammatory cytokine-coding gene *MIF* (macrophage migration inhibitory factor). The secretion of MIF from epithelia acts as a recruiting call to inflammatory cells, and has been shown to augment secretion of other pro-inflammatory cytokines (IL-8 and TNF), thereby prolonging the immune response (Lue *et al.*, 2002) and leading to exacerbation of underlying lung disease in susceptible individuals (Seaton *et al.*, 1995).

6.4.2.5 Apoptosis

The only gene altered in this group was *TRADD* (tumour necrosis factor receptor-signalling factor associated via death domain), following urban PM_{10} exposure. *TRADD* is a pro-apoptotic gene, yet also activates the NFκB signalling pathway (Hsu *et al.*, 1995); this may have indicated an inflammatory pathway response in the urban sample.

6.4.2.6 Microarray Technical Critique

Toxicogenomic technology is not without its drawbacks - arrays only measure relative, not absolute, levels of mRNA expression and changes in RNA do not necessarily correlate to a change in protein expression. It may also be difficult to differentiate between members of a gene family which have high sequence homology. In addition, the hypothesis-driven strategy employed in this study, of targeting a particular genetic response (toxicology and drug-resistance pathways), meant that whole genome changes from exposure to landfill PM_{10} were not investigated.

Even though stringent statistics was utilised in the SAM analysis, a greater number of replicates would provide greater accuracy of data. The combination of more replicates used in conjunction with a quantitative method such as qPCR to support results from the microarray analysis, would add support to subsequent findings. Although qPCR was not undertaken in the present study,

using the same SABiosciences microarray system, Sexton *et al.*, (2008) reported excellent agreement between qPCR and array data.

In contrast to *in vitro* monolayer cultures, the EpiAirway™ model is a more accurate representation of human respiratory morphology, thereby providing a human end-point in exposure studies; unlike animal-derived data, which requires extrapolation to human responses. The drawbacks of the system are its expense and small window of viability during which time the cultures can used in experiments (48h maximum). This might have led to acute responses to PM exposure being missed. Future work should be designed to follow time-course assessment (e.g. 6h), since other investigators have reported a temporal gene regulation, following laboratory animal instillations with reference urban PM (Kooter *et al.*, 2005).

6.5 CONCLUSIONS

The PSA confirmed the year-on-year significant oxidative bioreactivity of the smallest sized urban PM ($PM_{2.5-0.1}$), but only the landfill $PM_{2.5-0.1}$ collection 2007 exhibited similar deleterious effects upon the plasmid DNA; this indicated heterogeneity in the PM emitted at the landfill waste site. The $PM_{10-2.5}$ samples were relatively inert. The oxidative capacity of the $PM_{2.5-0.1}$ was retained exclusively in the soluble fraction, corresponding to comparatively high transition metal content (Chapter 5). The discovery of SELF and metal chelator-dependent amelioration of bioreactivity confirmed transition metals potentiated ROS generation from the most bioreactive samples of the PSA model.

The EpiAirway™ exposure indicated neither landfill nor urban PM_{10} were cytotoxic. Nonetheless, microarray analyses indicated a disruption to cellular homeostasis, predominantly centred on endoplasmic reticulum stress; in comparison, exposure to urban PM_{10} elicited a similar cellular stress, with an additional anti-oxidant response to ROS generation, possibly from the particulate matter.

CHAPTER 7

GENERAL DISCUSSION

7.1 OVERVIEW

Human activities have always generated waste, however the amounts and variety of waste generated has dramatically increased in relatively recent human history (OECD, 2008). A number of high-profile pollution episodes have led to public mistrust in waste management activities. Purported adverse public health outcomes include reproductive, respiratory, dermatological, cancers, and host of subjective symptoms (e.g. headaches and nausea). One such study involved landfill C: the Nant-y-Gwyddon waste site, where gastroschisis (a congenital birth defect) was found to be significantly elevated post-landfill operation (Fielder *et al.*, 2000). Although difficult to ascertain due to the long latency period and population migration, the risk of stomach, liver, prostrate, uteri and lung cancers was found to be elevated at the (now inoperative) Miron Quary waste site; once the 3rd largest landfill in North America (Goldberg *et al.*, 1995). In both studies, landfill gas was the principal emission of concern (ATSDR, 2001, 2002). The majority of suspected pollution episodes were industrial incidents and epidemiological studies were often inconclusive, nonetheless, a prevalent mentality of *"not in my back yard"* (NIMBY) now exists (Giusti *et al.*, 2009). Despite the concern surrounding adverse human health effects and landfills, our current understanding of causality is limited due to the observational, retrospective nature of most epidemiological studies (Redfearn and Roberts, 2002; Rushton, 2003) and the lack of long-term field measurement data.

The overall objective of this research was to investigate the bioreactivity of landfill leachate and airborne particulate matter (PM_{10}) emissions, in order to assist future research programmes with how to determine the level of environmental risk potentially posed to human health. Due to the limitations of analytical and human resources, it was beyond the scope of this project to prove or refute the causality between adverse human health effects and landfill leachate or PM_{10}. Nonetheless, the preliminary data here suggested that both landfill and urban PM_{10} were capable of generating free radicals. The bioreactivity of these emissions was considered in conjunction with the derived physicochemistry. There has been a lack of information regarding the metal-

driven, free radical and ROS-generating capacity of landfill leachates and PM_{10} (Li *et al.*, 2005; Bortolotto *et al.*, 2009). Environmental stressors are a known contributor to human health and disease, including cancer, cardiovascular and chronic pulmonary diseases, diabetes and neurodegeneration (González-Flecha, 2004; Valko *et al.*, 2007). Increasing evidence now exists to suggest the redox state is crucial in the development of these conditions (Figure 7.1; Franco *et al.*, 2009). Hence, this current study aimed to provide evidence that potential human health effects may be driven by the oxidative capacity of landfill emissions.

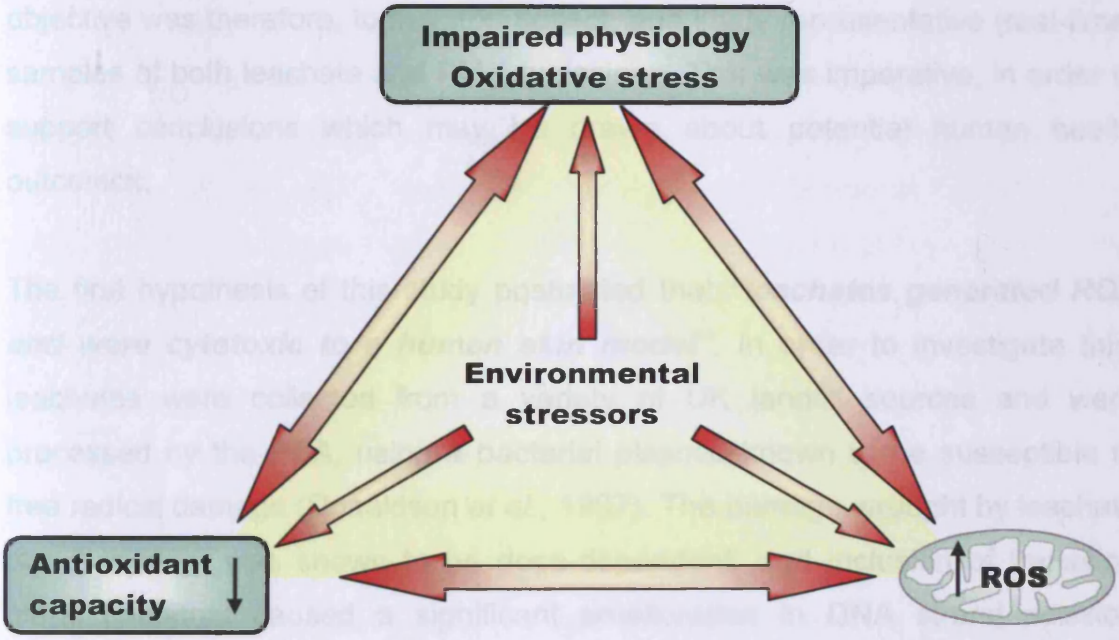

Figure 7.1: *Exposure to environmental toxicants may trigger various mechanisms which result in depletion of cellular antioxidant defences (enzyme function and the antioxidant pool), leading to oxidative stress and promotion of adverse health effects.*

A review of the literature revealed landfill leachate was predominantly investigated in terms of general physicochemistry and ecotoxicity, yet very few reports focussed on the oxidative capacity of landfill leachate as a possible mechanistic explanation of the toxicological effects. The metal component of the PM emissions was selected as the main focus of the chemical analyses, since there has been strong empirical evidence that this inorganic fraction influences ROS generation (Wilson *et al.*, 2002; Tao *et al.*, 2003; Risom *et al.*, 2005).

Very little source-specific information on the chemistry or toxicity of landfill PM_{10} exists. In particular, size-specific characterisation has been absent from the scientific literature. The logistics of landfill sampling are often restricting and unsurprisingly, few reports of potentially hazardous ambient landfill PM have been found in the literature. For example, Vega *et al.*, (2001) obtained landfill PM_{10} from re-suspension and filtering of waste mass surface sweepings; this may not be considered an accurate representation of airborne landfill PM_{10}. Other investigators have focussed collections at MSW transfer stations, where the anthropogenic activities would be quite different to that of an operating MSW landfill (Gladding, 2002; Baker *et al.*, 2003). An important research objective was therefore, to monitor, collect, and study representative (real-time) samples of both leachate and PM_{10} emissions. This was imperative, in order to support conclusions which may be drawn about potential human health outcomes.

The first hypothesis of this study postulated that ***"leachates generated ROS and were cytotoxic to a human skin model"***. In order to investigate this, leachates were collected from a variety of UK landfill sources and were processed by the PSA, using a bacterial plasmid known to be susceptible to free radical damage (Donaldson *et al.*, 1997). The damage wrought by leachate in this system was shown to be dose-dependent, and inclusion of transition metal chelators caused a significant amelioration in DNA strand scission (Koshy *et al.*, 2007). Taken together, these findings indicated that landfill leachates were capable of generating cell-free ROS. Since occupational dermal exposure was the most likely exposure route in the UK (Gray, 2005), landfill leachates were introduced to the apical side of a human skin tissue equivalent (EpiDerm™), for a single-exposure (24h period). As no observable adverse effects upon issue integrity and cell viability were noted, the lack of cytotoxicity suggested that the nature of the human epidermis was a robust barrier to incidental landfill leachate exposure.

In parallel to the above experimental methodology, an avenue regarding the toxicity of landfill leachates to *V. fischeri* (ROTAS™) was explored. Since the *V. fischeri* model has often been used during environmental monitoring,

investigating the relationship between the PSA and the ROTAS™ was considered beneficial to the selection of an assay for environmental screening. However, no obvious correlation was found between the two systems. Instead, the results revealed temporal hormesis responses in *V. fischeri* from several landfill leachates; cautioning against the unreserved use of pre-programmed toxicity testing kits.

The second hypothesis to be examined was that ***"physicochemical characteristics of landfill PM_{10} differed to urban PM_{10}, thereby affecting the bioreactivity"***. This was certainly found to be the case when two batches (2007 and 2008) of waste mass airborne PM sampling was undertaken. This protocol also revealed that whereas urban PM_{10} derived from vehicular emissions, and exhibited a consistent physicochemistry and bioreactivity profile, the landfill PM_{10} demonstrated significant temporal (year-on-year) variations in the masses emitted, and the metal and anion contents. The initial landfill sample collected in 2007, presented a similar traffic-related chemical signature to the urban collection, which manifested as favourably comparable levels of soluble transition metals and anion content. Both landfill and urban 2007 $PM_{2.5\text{-}0.1}$ samples were highly oxidative in the PSA, and the use of metal chelators ameliorated this activity, supporting the role of transition metals in PM_{10} oxidative capacity. In contrast, the second (2008) landfill sampling campaign, performed during anthropogenic road-dust entrainment, generated extremely large masses of poorly soluble aluminosilicate mineral matter. Since the radical-generating capacity was found to reside in the soluble portion of the PM, the 2008 landfill collection exhibited poor activity and this was classed as a nuisance pollutant; predicted to be cleared by the extra-thoracic protective mechanisms (coughing, sneezing and mucociliary defence; Mohanraj and Azeez, 2004).

In the final phase of bioreactivity assessment, the 2007 collections of landfill and urban PM_{10} were applied to the apical surface of a human tissue equivalent of the respiratory epithelium (EpiAirway™), for a single-exposure (24h period). The EpiAirway™ dose concentration of 50μg/cm^2 was chosen to represent a high-dose, high-risk individual (Phalen *et al.*, 2006). Such individuals could be

assumed to have an underlying lung disease (which would cause uneven airway ventilation), increased oral breathing (which would by-pass the nasal clearance mechanisms) and be in close proximity to the PM source (maximal exposure). Although no differences in EpiAirway™ tissue integrity and cell viability were observed, the genomics revealed a significant up-regulation of several "stress response and toxicology" genes from both landfill and urban PM_{10} (Koshy *et al.*, 2009).

7.2 CONCLUSIONS

The wide-ranging, multi-disciplinary examination of landfill emissions meant that several findings came to light, and these have been discussed below, within the context of landfill leachate or PM_{10}.

7.2.1 LANDFILL LEACHATE

Key deductions from ROS-sensitive bioreactivity assays:

- The PSA appeared to exhibit a threshold dose-response, indicating it was suitable as a viable screening tool for leachate bioreactivity

- The free radical-sensitive PSA was supported by the ROS-sensitive DCFH assay, and this combination could be used as a cell-free component in a battery of assays

- In both the PSA and DCFH systems, the response was ameliorated following metal-elimination either by Chelex resin treatment or by inclusion of metal chelators in the reaction mix, supporting the role of metal-induced free radicals in leachate bioreactivity.

Key deductions from EpiDerm™ leachate exposure:

- The exposure of the human tissue equivalent, EpiDerm™ to freshly generated leachate from all three landfills did not elicit cytotoxicity. This was suggested by the lack of adverse effect in either the TEER or cell viability assay

- These conclusions stand true for single, 24h exposures and were not comparable to repeated exposures, commonly used in skin-irritancy tests, which may induce alternate biochemical pathways.

Key deductions from leachate physicochemical analysis:

- The use of landfill-operator chemical data did not reveal any significant correlations between the leachate metal content and PSA bioreactivity. This may have been due to the differences that existed in the sampling regime of individual operators, such as non-refrigerated storage prior to analysis (Barnett, A., personal communication, 2008)

- Although the chemical analyses were conducted by UKAS-certified laboratories, there were a number of missing values from the dataset. This weakened the strength of the statistical analysis, restricting the conclusiveness of the physicochemical investigation. The varying reporting-requirements placed by the Environment Agency upon each of the landfill operators would benefit from standardisation.

Key deductions from leachate toxicity to *V. fischeri*:

- The majority of the leachates caused hyper-bioluminesence of *V. fischeri*. To effectively monitor this, incubation was extended to 100min using a research version of the ROTAS™ software. The simple "traffic-light" reporting system of the ROTAS™ commercial version software (used in pilot studies) gave misleading data and prevented the identification of hormetic responses. Caution must be exercised when using such kits for regulatory monitoring

- Since an "environmentally clean" groundwater sample also elicited a relatively mild β response, this could be taken into consideration and used as a baseline effect when reporting future hormesis profile data. There appeared to be little correlation between the PSA and ROTAS™

assays, suggesting leachate-sourced free radicals and ROS were not the primary protagonists of *V. fischeri* toxicity.

7.2.2 *LANDFILL PARTICULATE MATTER*

Landfill PM_{10} collections were obtained from consecutive years (2007 and 2008) and analysed using several tools (Figure 7.2). Since the PM_{10} for morphological analyses were obtained from different collection devices, temporal findings were presented and discussed with a note of caution. However, within each collection year, the comparisons between the landfill and urban samples were more conclusive.

Key deductions from PM_{10} morphological analysis:

- The landfill PM_{10} collections appeared to vary in particle size, number and mass measurements year-on-year. This conclusion was tempered by the bias of using two collection devices for morphological analysis

- Due to the significant biodegradable component of domestic waste (Sykes *et al.*, 2007), the absence of biogenic matter in either the 2007 or 2008 PM collections was an unexpected discovery. This may have been due to operator error in PM classification, or from the regulation-driven diversion of biomass away from landfill disposal (European Council Directive 1999/31/EC)

- Neither Negretti nor HVCI collectors provided the ideal PM_{10} collection substrate. In order to eliminate inter-device variation, the investigator recommends the use of a single collection substrate for characterisation and bioreactivity assays.

Key deductions from PM_{10} metal and anion analysis:

- A dominant Si, Al, Ca, Mg and Fe component in landfill PM_{10}, supported the morphological identification of crustal (aluminosilicate) minerals at the landfill, whereas the urban samples exhibited a strong S signal, lending support to the earlier morphological identification of urban combustion emissions

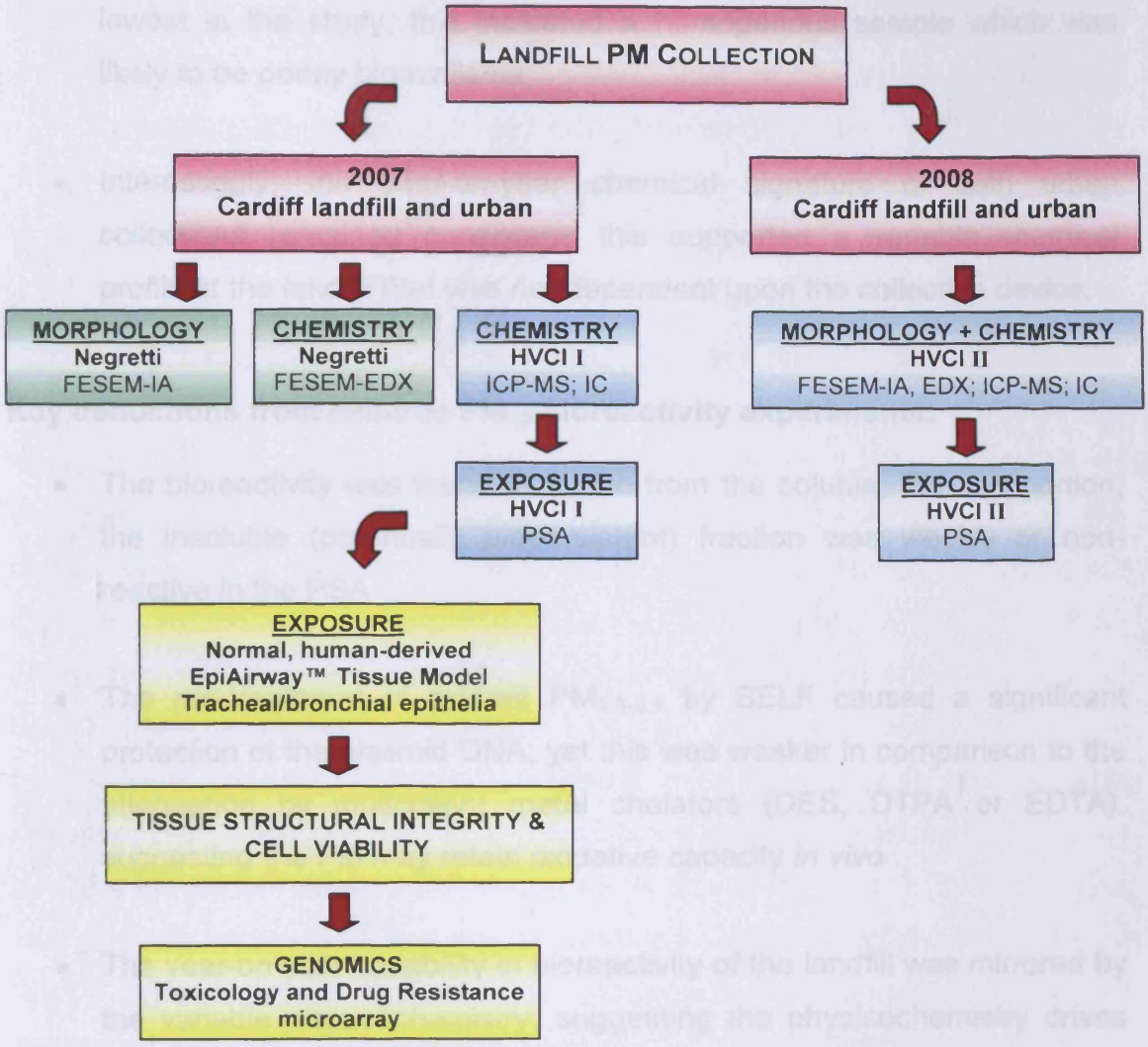

Figure 7.2: *Overview of the work schematic adopted for the investigation of landfill and urban PM_{10}. The Negretti collector was initially considered to generate samples ideal for SEM analyses; however, in an effort to avoid collector-bias and to standardise the methodology, a single collection substrate was used in 2008.*

- Landfill 2007 $PM_{2.5-0.1}$ was the only landfill sample to contain high concentrations of soluble redox-active metals (Cd, Cu, Co, V, Mn, Pb, Zn) and anions (Cl^-, SO_4^{2-} and NO_3^-), and showed significant overlap with the urban $PM_{2.5-0.1}$ chemical profile, suggesting similar source (vehicular) apportionment

- There appeared to be very little difference in the solubility of landfill 2008 $PM_{2.5-0.1}$ or $PM_{10-2.5}$. Since the soluble metal and anion contents were the

lowest in the study, this indicated a homogenous sample which was likely to be poorly bioavailable

- Interestingly, the year-on-year chemical signature of both urban collections remained consistent; this supported a variable chemical profile at the landfill that was not dependent upon the collection device.

Key deductions from cell-free PM_{10} bioreactivity experiments:

- The bioreactivity was found to derive from the soluble, $PM_{2.5-0.1}$ portion; the insoluble (potentially biopersistent) fraction was weakly or non-reactive in the PSA

- The pre-treatment of oxidant $PM_{2.5-0.1}$ by SELF caused a significant protection of the plasmid DNA; yet this was weaker in comparison to the attenuation by multivalent metal chelators (DES, DTPA or EDTA), suggesting the PM may retain oxidative capacity *in vivo*

- The year-on-year variability in bioreactivity of the landfill was mirrored by the variable physicochemistry, suggesting the physicochemistry drives the bioreactivity.

Key deductions from EpiAirway™ PM_{10} 2007 exposure:

- Neither landfill nor urban PM_{10} 2007 induced cytotoxicity in EpiAirway™ tissue

- Genomics (pre-validation) revealed 14 mRNA transcripts were up-regulated (≥3-fold) by landfill PM_{10}, and 12 transcripts were up-regulated (≥3-fold) by urban PM_{10}. Five of these were up-regulated in both treatments

- Since chaperones and heat shock protein genes were the major up-regulated functional group from landfill PM_{10} exposure, whilst drug

metabolism mRNA transcripts were the major up-regulated functional group from urban PM_{10} exposure, this might provide preliminary indicators of biomarkers of exposure from landfill PM_{10}.

7.3 FUTURE WORK

Since both landfill leachate and PM_{10} have now been linked to the generation of ROS via the PSA, alternate techniques which could confirm free radicals *in situ* would lend further support to this hypothesis. Although ICP-MS was a highly valuable tool in chemical profiling, it could not provide information on the oxidation states of metals. The valencies of metals are directly related to the generation of free radicals and ROS (Merolla and Richards, 2005; Valko *et al.*, 2005), hence it would be useful to identify which metal species of leachate or landfill PM_{10} (specifically, $PM_{2.5-0.1}$) were the driving force(s) behind the bioreactivity observed. In working towards this, the identities of candidate metals might be established if the post-chelated samples could be analysed by ICP-MS, but only once removal of the metal-ligand complex from the sample was achieved. Although this would be relatively simple for solid resin chelators (e.g. Chelex, DMG and ABO), the challenges of water-soluble chelator separation are more numerous and are currently under further investigation.

It would also be useful to identify the ROS species generated by the samples, such as evidence of the $^{\bullet}OH$ radical which has been implicated by the PSA. This could be achieved by use of electron spin resonance (ESR) or spin trapping of free-radicals (Shi *et al.*, 2003).

7.3.1 LANDFILL LEACHATE

The human skin is rich in AA and α-tocopherol (Cross *et al.*, 2002). Exposure of EpiDerm™ to leachates that were shown to be capable of generating free radicals (via the PSA and DCFH assays), did not result in measurable adverse effects. It would therefore be useful to examine the tissue for antioxidant status or evidence of oxidised protein (e.g. carbonyl derivatives) and lipid peroxidation (e.g. malondialdehyde). This might provide health effect biomarkers (Giusti *et*

al., 2009), of which there is a paucity in the current literature. An additional improvement would be performance of repeat-exposures, bringing the system into comparison with the scenario of contact dermatitis (Welss *et al.*, 2003), and which has been reported in landfill workers (Poulsen *et al.*, 1995; Kitsantas *et al.*, 2000). This could proceed with two sequential 24h applications of leachate onto EpiDerm™ before tissue harvest (Mun *et al.*, 2009). Since previous researchers have reported temporal variations in gene expression, shorter exposure times (e.g. 30min, 4h; Fletcher *et al.*, 2001) might also shed more light upon the defence mechanisms in this model of human skin.

As a commercially-sourced alternative to the ROTAS™ system, the MetPLATE™ toxicity testing kit (specific for the detection of metal toxicity in *V. fischeri*) might be used in future. The use of this system may be able to confirm or refute the phenomenon of hormesis exhibited by the microorganisms.

7.3.2 LANDFILL PM_{10}

The pre-treatment of PM_{10} with SELF, revealed significant amelioration of DNA strand scission in the PSA; it would be of great interest to quantify the change in antioxidant content of the individual components within the SELF fluid. Uric acid has been shown to be far less sensitive to metal-derived free radicals (Mudway *et al.*, 2004); hence a greater depletion of GSH and AA may provide a direct indication of the SELF efficacy. This could be conducted by HPLC of the post-incubated, particle-free SELF (Greenwell, 2003), and followed as concentration, and time-course experiments.

In a final statement regarding the EpiAirway™ genomics data presented in this study, the confirmation of these results by qPCR would be necessary to establish validity. Until then, these results can only be accepted as preliminary data. Further work should also aim to investigate shorter (e.g. 4h, 8h and 12h) exposures for early-response events (Koike *et al.*, 2004). Given sufficient financial resources, a whole-genome array could be used, given that the toxicology and drug resistance microarray represented less than 1% of the human genome.

Due to time and technical limitations, the current study did not make any attempt to quantify the contribution of organic compounds to landfill PM_{10}. Several investigations have confirmed the contribution of bioaerosols to landfill emissions, and carbonaceous matter as a well-known transporter of potentially detrimental organic compounds (Paoletti *et al.*, 2002). Although the current study failed to morphologically identify biological material, if provided with sufficient mass of landfill PM_{10}, it would be beneficial to establish PM-associated protein levels and the allergenic properties of PM_{10}. Recent publications on the effects of bioaerosols released during municipal compositing activity generally focus concern towards occupational exposure, where occurrences of "organic dust toxic syndrome", increased antibody levels and gastro-intestinal complaints are commonly reported (Poulsen *et al.*, 1995; Gladding, 2002; Sykes *et al.*, 2007). Recent epidemiological literature also maintains a positive association between bioaerosol emissions and irritant respiratory symptoms in nearby residents (Herr *et al.*, 2004). Establishing PM_{10} protein levels with a Bradford assay (Gutiérrez-Castillo *et al.*, 2005) might yield provisional information on respirable levels of endotoxin and microbial cell-wall fragments, known to induce or exacerbate the above-mentioned respiratory conditions (Sykes *et al.*, 2007). This could be further refined with an endotoxin-specific colorimetric kit assay (Menetrez *et al.*, 2007).

When dispersal processes were considered, the potential for occupational exposure of the waste site workers to landfill PM_{10} was likely to be greater than to the general public. The logistical challenges involved in sampling from the cabins of on-site vehicles meant that it was not possible to collect cabin PM samples with the current equipment. Therefore, if suitable equipment could be procured and specified to suit the workers and vehicular instability, occupational PM_{10} exposure might be further characterised. In addition to these personal-zone measurements, it would be useful to perform sampling campaigns within residential zones adjacent to, and downwind of MSW landfill sites, adding to a database for landfill exposure assessment. This could be further refined to residential locations within 2km of the waste site, as this distance has been quoted to be the likely dispersal limit of landfill emissions (Elliot *et al.*, 2001). It is also imperative that monitoring of landfill emissions are

performed on a long-term basis and the author recognised just two sampling campaigns were evaluated in this study.

7.4 ULTIMATE CONCLUSION

Establishing whether adverse human health end-points are caused by MSW landfill emissions is a hugely complex process, involving fields as diverse as toxicology, hydrogeology, meteorology, exposure risk assessments and epidemiology. The current study would benefit from consideration as part of a wider research programme, since identifying the mechanistic basis for postulated adverse health effects would inform the scientific rationale for landfill legislation and regulation.

MSW landfill emissions (leachate and PM_{10}) were collected from locations in south Wales, and their metal-mediated, oxidative, free radical generating capacity was indirectly confirmed by the (cell-free) PSA in conjunction with metal chelators and antioxidants. Despite this obvious oxidative capacity, these bioreactive samples were not cytotoxic to human tissue equivalents, and the genomics of PM exposure to the EpiAirway™ model concluded that the landfill PM_{10} were unlikely to present a greater hazard than urban PM_{10}. Although landfills are often viewed with public mistrust, the benefits of a well-regulated disposal system undoubtedly prevents human disease in the wider global context.

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APPENDICES

METAL (µg/g)	PM_{10} SIZE FRACTION	SAMPLE COLLECTION 2007			
		LANDFILL		URBAN	
		TOTAL	SOLUBLE	TOTAL	SOLUBLE
Al	$PM_{10-2.5}$	**34622**	< 4	6540	< 4
	$PM_{2.5-0.1}$	**4582**	< 4	415	< 4
Ca	$PM_{10-2.5}$	**150365**	**117683**	50615	41283
	$PM_{2.5-0.1}$	**42735**	**40483**	8815	7883
Na	$PM_{10-2.5}$	28125	19400	**130515**	**100350**
	$PM_{2.5-0.1}$	11955	20750	**40405**	**32250**
Mg	$PM_{10-2.5}$	**28572**	7350	19852	**14650**
	$PM_{2.5-0.1}$	5337	4450	**5804**	**5700**
Fe	$PM_{10-2.5}$	**43508**	**850**	10738	350
	$PM_{2.5-0.1}$	**7818**	1350	1838	**1750**
As	$PM_{10-2.5}$	**12**	**20**	< 0.4	< 0.3
	$PM_{2.5-0.1}$	**158**	**184**	< 0.4	< 0.3
Cd	$PM_{10-2.5}$	< 0.16	< 0.10	< 0.16	< 0.10
	$PM_{2.5-0.1}$	**13**	**12**	< 0.16	< 0.10
Cr	$PM_{10-2.5}$	**1161**	< 6	**960**	< 6
	$PM_{2.5-0.1}$	**933**	26	881	**131**
Cu	$PM_{10-2.5}$	**328**	53	61	**70**
	$PM_{2.5-0.1}$	**187**	177	< 3	**240**
Co	$PM_{10-2.5}$	**21**	< 0.04	6	< 0.04
	$PM_{2.5-0.1}$	**9**	**3**	4	0.65
Ni	$PM_{10-2.5}$	3055	< 26	**3325**	< 26
	$PM_{2.5-0.1}$	**3175**	44	3095	**99**
Pb	$PM_{10-2.5}$	**711**	< 1	101	< 1
	$PM_{2.5-0.1}$	**2599**	**1280**	378	222
V	$PM_{10-2.5}$	**106**	< 0.2	21	< 0.2
	$PM_{2.5-0.1}$	109	41	**177**	**124**
Mn	$PM_{10-2.5}$	**2847**	**356**	488	193
	$PM_{2.5-0.1}$	**923**	**607**	150	154
Zn	$PM_{10-2.5}$	**2062**	62	< 22	**149**
	$PM_{2.5-0.1}$	**8835**	**10064**	409	1021

Appendix A: *Total and water-soluble metals in µg/g of coarse and fine fractions of landfill and urban collections in 2007. Figures in bold font indicate highest total or soluble values within each size fractions. Undetected species indicated by <LOD.*

Metal (µg/g)	PM_{10} size fraction	Sample Collection 2008			
		Landfill		Urban	
		Total	Soluble	Total	Soluble
Al	$PM_{10-2.5}$	**44027**	**1525**	9893	< 4
	$PM_{2.5-0.1}$	**23677**	< 4	3978	< 4
Ca	$PM_{10-2.5}$	**118913**	35067	57883	**62083**
	$PM_{2.5-0.1}$	**75903**	**37767**	14793	9183
Na	$PM_{10-2.5}$	6440	< 259	**113267**	**129900**
	$PM_{2.5-0.1}$	8000	3625	**51595**	**33200**
Mg	$PM_{10-2.5}$	**26219**	2300	23205	**23500**
	$PM_{2.5-0.1}$	**13864**	3800	9789	**5500**
Fe	$PM_{10-2.5}$	**49366**	275	27241	**800**
	$PM_{2.5-0.1}$	**27026**	300	15614	**21650**
As	$PM_{10-2.5}$	7	< 0.4	2	< 0.4
	$PM_{2.5-0.1}$	12	< 0.4	**60**	**11**
Cd	$PM_{10-2.5}$	< 0.16	< 0.10	< 0.16	< 0.10
	$PM_{2.5-0.1}$	< 0.16	< 0.10	< 0.16	< 0.10
Cr	$PM_{10-2.5}$	**535**	**13**	227	6
	$PM_{2.5-0.1}$	**398**	< 6	< 70	**42**
Cu	$PM_{10-2.5}$	**218**	11	271	**90**
	$PM_{2.5-0.1}$	131	18	**440**	**250**
Co	$PM_{10-2.5}$	**18**	< 0.04	7	**1**
	$PM_{2.5-0.1}$	**12**	< 0.04	4	**3**
Ni	$PM_{10-2.5}$	**1915**	< 26	963	< 26
	$PM_{2.5-0.1}$	**1689**	< 26	1565	**68**
Pb	$PM_{10-2.5}$	**358**	< 0.02	51	< 0.02
	$PM_{2.5-0.1}$	350	< 0.02	**1006**	**442**
V	$PM_{10-2.5}$	**50**	< 0.2	2	< 0.2
	$PM_{2.5-0.1}$	**107**	< 0.2	99	**58**
Mn	$PM_{10-2.5}$	**2697**	46	871	**433**
	$PM_{2.5-0.1}$	**1533**	73	536	**406**
Zn	$PM_{10-2.5}$	**323**	11	301	**544**
	$PM_{2.5-0.1}$	582	6	**1998**	**2037**

Appendix B: *Total and water-soluble metals in µg/g of coarse and fine fractions of landfill and urban collections in 2008. Figures in bold font indicate highest total or soluble values within each size fraction. Undetected species indicated by <LOD.*

Gene ID	Gene name	Gene Family	Fold change	
			Landfill	Urban
NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)	Drug metabolism	2.8	n/a
CANX	Calnexin	Chaperones & HSP	2.5	n/a
CYP11B2	Cytochrome P450, family 11, subfamily B, polypeptide 2	Drug metabolism	1.6	n/a
HSPA9	Heat shock 70kDa protein 9 (mortalin)	Chaperones & HSP	1.7	n/a
DNAJC4	DnaJ (Hsp40) homolog, subfamily C, member 4	Chaperones & HSP	1.8	n/a
CCT8	Chaperonin containing TCP1, subunit 8 (theta)	Chaperones & HSP	3.4	n/a
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	Cell growth & proliferation	1.6	n/a
DNAJC5	DnaJ (Hsp40) homolog, subfamily C, member 5	Chaperones & HSP	1.6	n/a
BRCA1	Breast cancer 1, early onset	Cell growth & proliferation	1.9	n/a
HSPA1A	Heat shock 70kDa protein 1A	Chaperones & HSP	2.3	n/a
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	Drug metabolism	1.8	n/a
GSTM1	Glutathione S-transferase M1	Drug metabolism	1.6	n/a
FMO4	Flavin containing monooxygenase 4	Drug metabolism	1.6	n/a
BRCA2	Breast cancer 2, early onset	Transcription factor & regulator	1.8	n/a
DNAJC7	DnaJ (Hsp40) homolog, subfamily C, member 7	Stress response	1.6	n/a
E2F1	E2F transcription factor 1	Transcription factor & regulator	1.8	n/a
NR1I2	Nuclear receptor subfamily 1, group I, member 2	Transcription factor & regulator	1.7	n/a
HSPB1	Heat shock 27kDa protein 1	Chaperones & HSP	3.2	2.7
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Cell cycle	4.4	3.7
HSP90AA2	Heat shock protein 90kDa alpha (cytosolic), class A member 2	Chaperones & HSP	3.1	3.0
SOD2	Superoxide dismutase 2, mitochondrial	Drug metabolism	2.1	2.5
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	Cell cycle	3.2	2.5
HSP90AB1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	Chaperones & HSP	2.6	3.1
HYOU1	Hypoxia up-regulated 1	Chaperones & HSP	3.4	2.7
HSP90AB1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	Chaperones & HSP	2.8	2.6
HSPB3	Heat shock 27kDa protein 3	Chaperones & HSP	2.3	2.4
DNAJC8	DnaJ (Hsp40) homolog, subfamily C, member 8	Chaperones & HSP	2.5	1.8
TRADD	TNFRSF1A-associated via death domain	Apotosis	2.6	3.1
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	Cell prolif. & growth	3.0	3.2
NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)	Drug metabolism	2.8	3.6
ST13	Suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	Chaperones & HSP	1.8	2.2
DHFR	Dihydrofolate reductase	Drug metabolism	2.1	2.2
PON3	Paraoxonase 3	Stress response	2.5	2.4
RARA	Retinoic acid receptor, alpha	Transcription factor & regulator	1.9	2.4
NQO1	NAD(P)H dehydrogenase, quinone 1	Drug metabolism	4.5	4.1
MGMT	O-6-methylguanine-DNA methyltransferase	Drug metabolism	2.6	2.5
SOD1	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	Drug metabolism	2.7	3.8
HSF1	Heat shock transcription factor 1	Chaperones & HSP	2.0	1.7
NFKBIB	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, β	Transcription factor & regulator	2.7	2.1

Appendix C: *Differential gene expression (≥1.5-fold change compared to control) from toxicology and drug resistance microarray data following EpiAirway™ exposure to composite landfill or urban PM_{10}. Green coloured cells highlight the genes commonly expressed.*

GENE ID	GENE NAME	GENE FAMILY	FOLD CHANGE	
			LANDFILL	URBAN
CDK4	Cyclin-dependent kinase 4	Cell cycle	2.5	3.3
GPX1	Glutathione peroxidase 1	Drug metabolism	1.8	1.5
CHST6	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6	Drug metabolism	2.1	1.9
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Apoptosis	1.9	1.8
CYB5R3	Cytochrome b5 reductase 3	Drug metabolism	3.0	2.8
PRDX2	Peroxiredoxin 2	Drug metabolism	2.8	2.4
CCT8	Chaperonin containing TCP1, subunit 8 (theta)	Chaperones & HSP	3.4	5.1
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	Chaperones & HSP	1.7	2.0
HSPD1	Heat shock 60kDa protein 1 (chaperonin)	Chaperones & HSP	2.1	1.6
NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	Stress response	2.5	3.5
CDK2	Cyclin-dependent kinase 2	Cell cycle	2.3	2.6
PRDX1	Peroxiredoxin 1	Drug metabolism	4.4	3.7
MGST3	Microsomal glutathione S-transferase 3	Drug metabolism	2.5	3.3
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α	Transcription factor & regulator	4.8	2.8
NR1I3	Nuclear receptor subfamily 1, group I, member 3	Transcription factor & regulator	3.4	2.3
MGST2	Microsomal glutathione S-transferase 2	Drug metabolism	2.0	2.6
RELB	V-rel reticuloendotheliosis viral oncogene homolog B	Transcription factor & regulator	3.6	2.2
CHAT	Choline acetyltransferase	Drug metabolism	2.3	2.0
HSPA8	Heat shock 70kDa protein 8	Chaperones & HSP	1.7	1.7
DDIT3	DNA-damage-inducible transcript 3	Stress response	3.4	3.2
CCT3	Chaperonin containing TCP1, subunit 3 (gamma)	Chaperones & HSP	1.7	1.6
GSTO1	Glutathione S-transferase omega 1	Drug metabolism	2.6	2.2
BCL2L2	BCL2-like 2	Apoptosis	2.2	2.2
MYST2	MYST histone acetyltransferase 2	Transcription factor & regulator	2.0	2.0
TPST1	Tyrosylprotein sulfotransferase 1	Drug metabolism	2.4	2.5
GAL3ST1	Galactose-3-O-sulfotransferase 1	Drug metabolism	2.6	1.9
BCR	Breakpoint cluster region	Cell growth & proliferation	2.6	2.6
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	Cell cycle	1.6	2.0
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	Cell growth & proliferation	n/a	1.7
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	Transcription factor & regulator	n/a	2.6
CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)	Chaperones & HSP	n/a	1.7
CRYAA	Crystallin, alpha A	Chaperones & HSP	n/a	1.9

Appendix C concluded: *Differential gene expression (≥1.5-fold change compared to control) from toxicology and drug resistance microarray data following EpiAirway™ exposure to composite landfill or urban PM_{10}. Green coloured cells highlight the genes commonly expressed.*

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Bioreactivity of leachate from municipal solid waste landfills — assessment of toxicity

Lata Koshy [a,*], Emma Paris [b], Sarah Ling [b], Tim Jones [b], Kelly BéruBé [a]

[a] School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, Wales, UK
[b] School of Earth, Ocean and Planetary Sciences, Cardiff University, Museum Avenue, Cardiff CF10 3YE, Wales, UK

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Abstract

With the UK producing 400 million tonnes of waste each year, the problem of waste disposal is recognised as one of the most serious environmental problems facing the nation. Of this, over 35 million tonnes is municipal waste, largely derived from households, but also includes some commercial and industrial waste. There are strong national and international concerns about the possible adverse health effects of living in the vicinity of municipal waste landfills. An understanding of the ranges of toxicity of landfill emissions is crucial to determine the degree of concern we should have about the potential effects they could have upon nearby populations and the surrounding environment. Leachates from three different types of landfills have been collected and screened for their potential to induce toxicity. Bioreactivity was measured by a plasmid DNA scission assay (PSA), and 2′,7′-dichlorodihydrofluorescin fluorescence (DCFH). The results indicate that leachates cause damage to plasmid DNA in a dose-dependent manner and that toxicity varies between different types of landfills as well as within individual waste sites. Overall, the data implies that the complex chemistry involved in leachate formation has yet to be delineated in terms of the toxicological response.
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Keywords: Landfill leachate; Toxicity; *In vitro* assays; Municipal waste; Health effects

1. Introduction

An unavoidable issue, landfills are of great environmental importance in the UK, which currently disposes approximately 80% of its waste in landfill sites; placing the UK within the top 4 EU member landfillers (EC, 2005). High levels of air, water and soil contamination in a few well-publicised cases have led to an increasing number of epidemiological studies being carried out on the health risks posed by landfill sites (ATSDR, 2001; Berry and Bove, 1997; Fielder et al., 2000). However, the majority of these studies focus on illegal dumps and hazardous waste sites, with fewer investigations of municipal waste landfills. Landfills release a wide range of chemicals resulting from the waste degradation in the form of leachate, gas and particulate matter (PM), although the latter is only released from active landfills. The gas is collected, sometimes as a condensate due to the moisture inherent within municipal solid waste (MSW) landfills, and on-site drainage systems collect leachate. Modern landfills are built to be as self-containing as technologically possible, but it is accepted that all landfills will leak to some extent (McQuade and Needham, 1999). Landfill leachates are a complex mix of inorganic and organic components and combined with site specificity, often means that the route of exposure and toxicity remain unknown. Nevertheless, sites established prior to leachate

* Corresponding author. Tel.: +44 2920 876810; fax: +44 2920 875211.
E-mail address: KoshyL@cardiff.ac.uk (L. Koshy).

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management techniques and the potential of all landfills to release indeterminate volumes of leachates can be considered to constitute a public health hazard, as migration of pollution episodes could potentially compromise groundwater and surface water sources.

Leachate is generated by the percolation of rainwater and moisture through the layers of waste in landfills. This liquid migrates down through pores within the waste mass, and in modern containment landfills, drains away in the engineered drainage layer; collecting at the lowest point in a sump or storage reservoir. In the UK waste management industry, licenses for discharge consent of landfill effluents are based on the physicochemical parameters e.g. biological oxygen demand (BOD), pH, chemical oxygen demand (COD), total dissolved solids (Defra, 2002). However, these do not give an indication on the toxic potential of the leachate. With 80% of the UK population living within 2 km of a landfill (Elliott et al., 2001), there is great interest in the potential adverse health risks posed by such sites and their emissions, i.e. leachate, gases and PM. Leachates have been previously shown to induce abnormal sperm morphology (Bakare et al., 2005) and chromosomal abnormalities in bone marrow in mice, indicating that landfill leachate is genotoxic in mammalian cells (Sang and Li, 2005). Ecotoxicological studies have resulted in detrimental effects being observed in rodent and aquatic species but the extrapolation of these model systems to the human population requires multidisciplinary consideration (Bloor and Banks, 2005; Rutherford et al., 2000). This study focuses upon the bioreactivity and *in vitro* toxicity of leachate produced by three MSW landfills: active and containment landfill A; dilute and disperse landfill B; containment (now inoperative) landfill C, all located in south Wales.

2. Methods

2.1. Landfill sampling sites and collection methodology

The three waste disposal sites are situated within 25 km from Cardiff (Fig. 1), south Wales, and have been selected due to their different characteristics. Landfill A, a containment landraise site, and landfill B, a dilute and disperse landfill site, are both currently accepting MSW. Both A and B have accepted hazardous waste in the past including motor vehicle parts, tyres, and clinical waste; landfill B was originally used for industrial waste from the steel industry before its use for MSW. Landfill A is located within Cardiff city limits whilst landfill B is situated in a rural south Wales valley. Landfill C is a

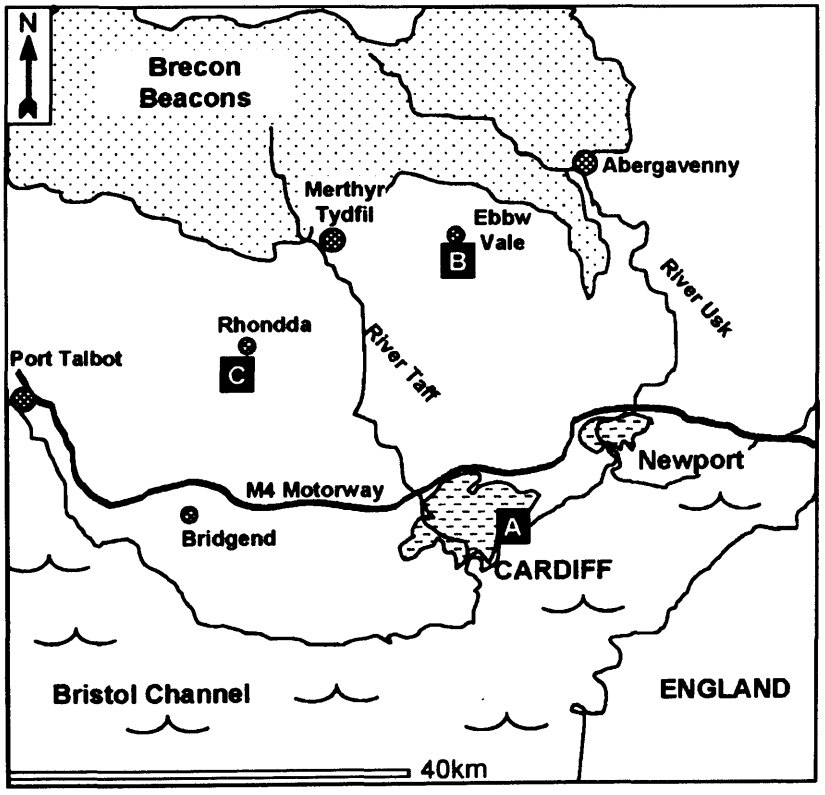

Fig. 1. Landfill sites of interest in south Wales. A = active contained site; B = active, dilute and disperse site; C = restored and contained site.

Table 1
Sampling well characteristics of landfill sites

Site	Type	Operation since	Waste type (in addition to MSW)	Collective Sump	Restored Phase	Active Phase	Leachate Reservoir	Leachate volume discharged (m^3/year)
A	Active; contained	1978-	Mixed Industrial, Chemical, Bulky	A1	A2	A3	-	200,000*
B	Active; dilute and disperse	1981-	Iron & Steel Industrial	B1	B2	-	B3	250,000*
C	Restored contained	1988-2002	Industrial, Filter cake	C1	C2	-	C3	36,250

* Estimated values based on landfill operator data during 2006.

containment landfill site built in a depression on the side of a rural south Wales mountain and no longer accepts waste. Leachates were sampled from collective sumps where leachate accumulates before site discharge, restored (capped) phases, active phases and leachate reservoirs where possible (Table 1) from November 2005 to October 2006.

All leachate samples were collected in 250 ml HDPE bottles with minimal headspace and filtered through mixed cellulose ester 0.45 μm membranes (Fisher Scientific, UK) before being stored at 4 °C in the dark until use.

2.2. Analytical techniques

2.2.1. Physicochemical analysis

All data were provided by the landfill operators after sample analysis by contracted and certified laboratories. The parameters assessed in this study include Ammoniacal-nitrogen (NH_4^+–N), pH, COD, BOD, suspended solids (SS), Cl, Mecoprop (MCPP), m&p-xylene and metals (Cd, Ni, Cr, Zn, Cu, Fe and Pb). Ion chromatography was performed at Cardiff University to analyse samples for Cl where data were not available.

2.2.2. Plasmid scission assay

The plasmid ΦX174 RF DNA molecule (Promega, London, UK) selected for use in the plasmid scission assay (PSA) has been shown to be susceptible to damage by ROS and metals which convert the supercoiled, undamaged isoform to relaxed and linear isoforms (Greenwell et al., 2002; Moreno et al., 2004). Filtered leachate samples were diluted in molecular grade water (Sigma-Aldrich, UK) and incubated with 200 ng ΦX174 RF DNA in a final volume of 20 μl. Negative controls were established using incubations of DNA in molecular grade water and positive control consisted of restriction enzyme Pst I (Promega, London, UK) digest. Samples were gently agitated for 6 h before 3.33 μl loading dye (Promega, London, UK) was added. Samples were electrophoresed on a 0.6% agarose 0.25% ethidium bromide gel for 16 h at 30 V in 1 x Tris-Borate-EDTA buffer. Electrophoresed gels were imaged using VisionWorks® software (Ultraviolet Products Ltd., UK) as depicted in Fig. 2 and densitometric analysis was performed with Genetools® software (Syngene® systems, UK).

The relative amount of damaged DNA (relaxed open circle form plus linearized form) in each lane was calculated as a percentage of the supercoiled intact DNA. Gels with less than 20% damage in the water controls were accepted for analysis. Statistical analysis included the Anderson–Darling normality test and Spearman's rank correlation provided in Minitab 14 (Microsoft Inc, WA, USA) and significant differences were calculated using a two-tailed t test; p values lower than 0.05 were considered to indicate statistical significance. The data was fitted to a non-linear regression sigmoidal model using GraphPad Prism 2 (GraphPad Software Inc, CA,

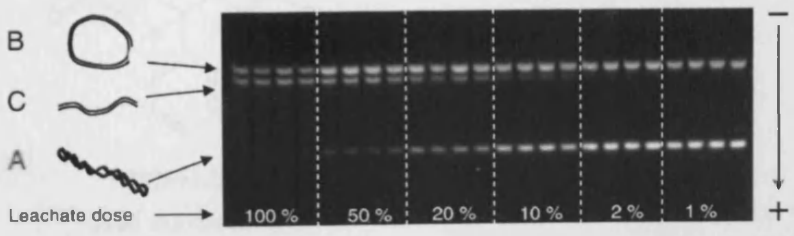

Fig. 2. Plasmid scission assay of ΦX174 RF DNA incubation with various concentrations of landfill leachate. Conformations at varying levels of progressive damage; A: Undamaged supercoil, B: preliminary damaged relaxed coil, C: severely damaged linear DNA.

USA). The concentration of leachate to induce 50% damage (TD50) was determined in order to facilitate comparison between samples. All curves were plotted ± SEM (n=4–6).

2.2.3. Dichlorodihydrofluorescin assay

Dichlorodihydrofluorescin (DCFH) was used as a probe for oxidant species. The compound was sourced as 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) (Sigma-Aldrich, UK) due to the increased stability of the precursor which was chemically cleaved to the working form of DCFH as indicated in Fig. 3. This compound has been widely used as a marker of cellular oxidative stress and as an indicator of reactive species formation (Gomes et al., 2005; Hung and Wang, 2001). A working solution of DCFH was prepared from DCFH-DA using the method of Cathcart et al. (1983). In brief, 2 ml 0.01 N NaOH was added to 0.5 ml 1 mM DCFH-DA in methanol and this hydrolysate was allowed to react at RTP in the dark for 30 minutes prior to neutralisation with 10 ml 25 mM NaH_2PO_4, pH 7.4 to provide a 40 μM stock solution of activated DCFH. Reactions were carried out at 37 °C for 25 min in light-resistant eppendorfs, containing 750 μl undiluted leachate and a final concentration of 1 μM activated DCFH.

Fluorescence intensity was measured in a Cary Eclipse Fluorimeter (Varian Instruments, CA, USA) with excitation wavelength at 485 nm and emission wavelength at 530 nm (slit width 10 nm). Measuring the fluorescence of each pre-incubation confirmed the absence of interfering organic compounds in leachate samples. All samples emitted background levels of fluorescence prior to addition of DCFH. Parallel incubations were performed with 1.485 ml 25 mM NaH_2PO_4, pH 7.4 containing a final concentration of 1 μM activated DCFH. This provided a measure of the extent of dye auto-fluorescence.

In order to correlate the fluorescence of samples to equivalents of H_2O_2, a linear calibration curve of H_2O_2 was generated. All calibration reactions were performed in 40 mM Tris-HCl pH 7.4 containing 1 μM activated DCFH solution. This mixture was spiked with 0.1–300 μM H_2O_2 before initiation of the reaction by addition of 10 μM $FeSO_4$ in a final volume of 1.5 ml.

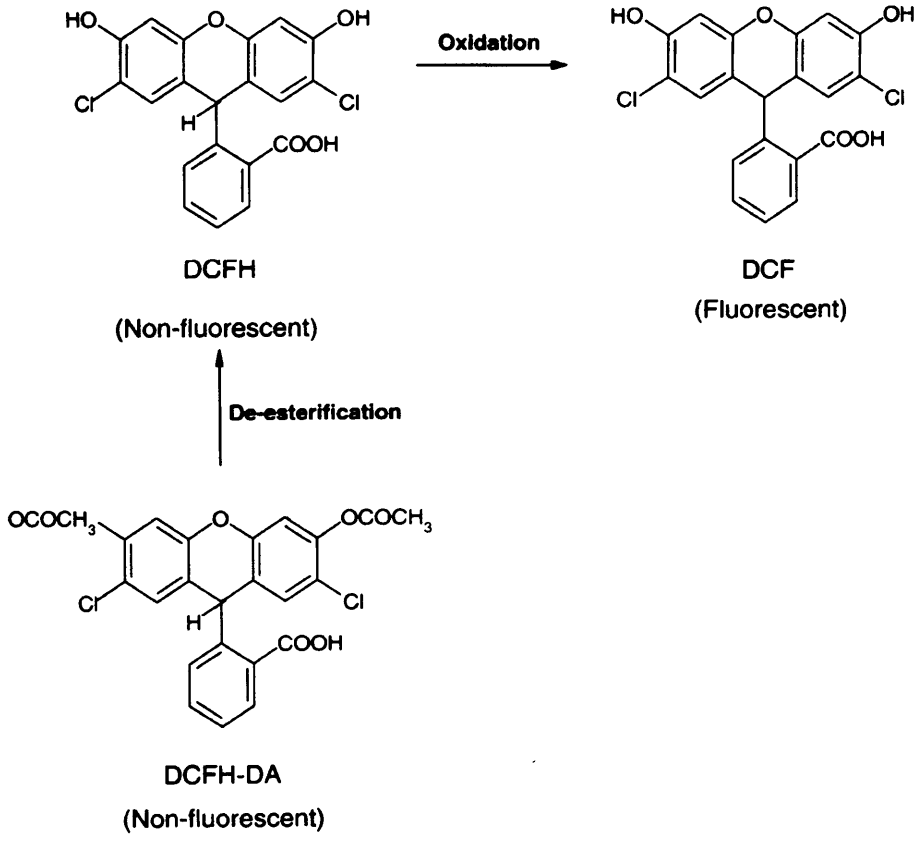

Fig. 3. Chemical activation of 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) to 2′,7′-dichlorodihydrofluorescin (DCFH) and the *in vitro* formation of the fluorogenic 2′,7′-dichlorofluorescin (DCF) moiety.

3. Results

3.1. Physicochemical analysis

The results for basic physicochemical analysis of the leachate samples are presented in Tables 2 and 3. Data for NH_4^+–N, pH, COD, BOD, SS, Cl, MCPP and m&p-xylene were acquired for the majority of leachates assessed by PSA. Metals analysed included Cd, Ni, Cr, Zn, Cu, Fe and Pb. The relationship between these parameters and toxicity as measured by the PSA was investigated with Spearman's rank correlation analysis and a significant negative association was observed between TD50 and MCPP, with a correlation coefficient of -0.817.

3.2. Plasmid scission assay

Not all dose–response sigmoidal curves for all leachates are presented, as trends for all samples were similar. In order to compare PSA results of different samples, a non-linear dose–response model was established for all samples (Fig. 4). The majority of samples were assigned TD50 values, but where sample maximum toxicity fell below 50%, a TD25 value was generated as listed in Table 2.

3.2.1. Landfill A (active and contained site)

Landfill A winter leachates revealed a significant temporal and spatial variation in toxicity with a general reduction in toxicity observed over the sampling period (Fig. 5). The exception to this was seen in the collective sample of active phase leachate, A1, collected during January. This was some of the least toxic leachate collected during the study (TD50:100%). The subsequent A1 leachate collected in February was significantly increased in toxicity (TD50:7%) before a reduction in toxicity as summer progressed, to a maximum TD50 value of 41% observed in August. The toxicity of A1 increased in September, before falling once again in October. The log EC50 (percentage of DNA damage at 50% leachate concentration) values of dose–response curves of A1 and A3 February and October leachate samples were compared for similarity by unpaired t test, which revealed no significant difference between the wells (data not shown). These samples also appear to be some of the most toxic leachates from landfill A (TD50:7% and 4% respectively). Although located in the restored phase of the landfill, A2 exhibited similar toxicity to A1 in the spring (April TD50: 9% and 8.5% respectively) and early summer (June TD50: 23% and 16%) before a significant fall in A2 toxicity ensued, with only TD25 values being generated for the remainder of the sampling period.

3.2.2. Landfill B (dilute and disperse site)

Landfill B appeared to exhibit distinct spatial variation in PSA toxicity associated with the different wells during the winter in the first half of the sampling duration, and although B1 and B2 followed similar trends during this period, there was a notably similar toxic response between these two wells' toxicity during the later summer months (Fig. 6). Overall, the storage reservoir, B3, leachate samples exhibited the greatest toxicity, whilst B1 and B2 were less toxic. The most toxic landfill B leachate was obtained from B3 in February (TD50:6%). A progressive fall in this well's toxicity was then observed, to TD50:35% in April followed by a period of fluctuating toxicity for the remainder of analysis. The most toxic B2 leachate was collected at the start of sampling in November (TD50:46%) after which a progressive weakening in toxicity was observed, leading to the least toxic B2 samples being collected in March and April, with the generation of TD25 values highlighted in grey, Fig. 6. Leachate from B1 analysed for PSA toxicity also revealed a weakening in toxicity from November to February (TD50: 48% to 94% respectively). B1 leachate collected in June was significantly more toxic than that of B2 leachate (TD50:51% and 81% respectively) but yielded similar TD50 values until the end of the sampling period.

3.2.3. Landfill C (restored and contained site)

Leachates collected from Landfill C exhibited a wide range of toxicity. The most toxic landfill C leachate was obtained from C1 in March, (TD50:1%), which was the most toxic leachate from any landfill collected during the study. Earlier sampling of C1 in January had demonstrated lower toxicity (TD50:45%), indicating an increase in C1 toxicity from January to March. Seasonal sampling of leachate from this well revealed a varying response of toxicity. The least toxic landfill C leachate was collected from C2, yielding a TD25 of 12% in August. In contrast to the seasonal trend elicited by C1 leachate, the TD50 of C2 leachate had risen from 8% in January to 97% in March, indicating a weakening in toxicity. However, the fluctuating trend in toxic response seen with leachate C1 was mimicked by leachate C2, with toxicity increasing to TD50:35% in July before the toxic response was reduced in August, leading to extrapolation of the only TD25 value from landfill C. The effluent collected from C3 yielded TD50 values of 26% in January and 57% in March, indicating a fall in reservoir leachate toxicity as winter progressed. (Fig. 7)

Table 2
Bioreactivity, physical and chemical characteristics of the leachate samples listed in order of decreasing PSA toxicity

Landfill leachate	Month	PSA TD25 leachate %	PSA TD50 leachate %	Equivalent H_2O_2 μM	NH_4^+–N mg/L	pH	COD mg/L O_2	BOD mg/L O_2	SS mg/L	Cl mg/L	MCPP** μg/L	m&p xylene μg/L
C1	March	–	1	182	1230	8.1	2640	34	68	1120	–	–
A1	September	–	2	290	–	6.9	–	–	–	945*	26.90	11.90
A3	February	–	4	45	606	–	–	–	–	812*	–	–
B3	February	–	6	291	1.21	8.6	–	19	58	586	–	–
A1	February	–	7	85	589	–	–	–	–	922	30.30	25.50
C2	January	–	8	154	–	–	–	–	–	1102*	–	–
A1	April	–	8.5	66	704	–	–	–	–	1270	25.30	24.80
A2	April	–	9	95	1	–	–	7	–	–	–	–
B3	May	–	10	325	88.8	8.3	–	9	12	229	–	–
A1	June	–	16	260	658	7.0	–	–	–	876	25.70	20.40
B3	March	–	17	124	1.68	8.4	–	19	6	474	–	–
A2	February	–	18	52	1.1	–	–	1	–	146*	–	–
C1	September	–	18	86	1240	7.6	2520	196	72	1340	–	–
C2	September	–	18	156	–	7.0	–	–	–	1206*	–	–
A1	October	–	20	84	367	6.8	–	–	–	487	–	6.31
A3	October	–	21	67	427	7.2	–	–	–	1023*	–	–
C1	June	–	22	70	840	8.1	2780	–	67	1300	–	–
A2	June	–	23	69	–	7.5	75	1	148	26*	–	–
C1	July	–	23	94	–	7.4	–	–	–	–	–	–
C2	October	–	23	–	–	7.2	–	–	–	1081*	–	–
B3	June	–	26	63	1.13	8.5	–	–	31	631	–	–
C3	January	–	26	44	91.6	8.2	291	17	452	193	–	–
B3	July	–	27	72	327	8.5	–	–	26	774	22.20	–
A1	July	–	28	55	597	7.2	–	–	–	840	27.40	23.70
B3	October	–	33	–	156	8.4	–	–	18	451	10.80	–
B3	April	–	35	–	361	8.5	–	–	84	633	21.00	–
C2	July	–	35	72	–	7.2	–	–	–	1465	–	–
C3	July	–	39	–	–	8.5	536	14	266	554	–	–
A1	August	–	41	51	–	7.0	–	–	–	45	18.70	13.70
C1	January	–	45	43	650	8.2	1860	211	–	952	–	0.96
B2	November	–	46	56	140	8.0	–	15	19	413	–	–
B3	August	–	47	86	359	8.5	–	15	26	780	–	–
B3	September	–	47	–	347	8.5	–	15	26	736	–	–
B1	November	–	48	–	207	7.0	–	28	32	529	–	–
B1	June	–	51	62	248	8.6	–	22	57	708	–	–
C3	August	–	51	–	307	8.2	657	22	478	644	–	–
C1	May	–	53	46	1290	8.0	2580	203	16	1330	–	16.60
C3	March	–	57	14	57.7	8.1	128	10	19	173	–	–
B2	January	–	58	34	114	8.3	–	8	25	336	–	–
C3	May	–	59	10	0.7	8.0	156	20	46	98	–	–
C1	August	–	64	65	1430	7.9	–	–	64	1290	–	–
B2	February	–	70	6	6.5	8.3	–	8	19	338	–	–
B1	August	–	76	20	353	8.5	–	15	44	833	–	–
B1	July	–	81	53	306	8.5	–	20	31	772	21.60	–
B1	September	–	81	–	390	8.5	–	18	38	814	–	–
B2	June	–	81	–	140	8.4	–	17	20	389	–	–
B2	July	–	81	46	121	8.3	–	13	15	410	10.20	–
B2	August	–	81	58	124	8.3	–	11	40	474	–	–
B2	October	–	82	6	76.6	8.0	–	4	9	206	4.64	–
B2	September	–	83	5	80.4	8.1	–	–	40	243	–	–
C2	June	–	91	–	–	7.4	–	–	–	1500	–	–
B1	February	–	94	2	0.89	8.6	–	17	42	637	–	–
C2	March	–	97	–	–	–	–	–	–	–	–	–
A1	January	–	100	70	331	–	–	–	–	580	–	–
B1	October	–	100	–	1.58	8.5	–	9	24	546	15.20	–
C2	August	12	–	–	–	7.2	–	–	–	1388	–	–
B2	April	32	–	–	106	8.3	–	10	28	–	–	–

Table 2 (*continued*)

Landfill leachate	Month	PSA TD25 leachate %	PSA TD50 leachate %	Equivalent H_2O_2 μM	NH_4^+–N mg/L	pH	COD mg/L O_2	BOD mg/L O_2	SS mg/L	Cl mg/L	MCPP** μg/L	m&p xylene μg/L
A2	August	34	–	–	–	7.3	–	–	–	82	–	–
A2	September	39	–	–	–	7.5	–	–	433	–	–	–
B2	March	49	–	19	–	8.4	–	–	7	192	–	–
A2	July	57	–	–	–	7.3	–	–	–	76	–	–
A2	October	84	–	–	–	6.9	–	–	–	78	–	–

* Analysis performed independently of landfill site operators.
** Mecoprop.

3.3. DCFH assay

A selection of leachate samples assessed by PSA was evaluated for reactive species by the formation of DCF from DCFH and a non-parametric correlation between PSA toxicity and equivalent H_2O_2 production was observed (Spearman's rank correlation (r_s)=−0.723, $p<0.05$) as depicted in Fig. 8.

Table 3
Metal characteristics of the analysed leachate samples

Landfill leachate	Month	PSA TD50 leachate %	Cd μg/L	Ni mg/L	Cr mg/L	Zn mg/L	Cu mg/L	Fe mg/L	Pb mg/L
A1	September	2	–	0.120	–	–	–	–	–
A3	February	4	5	–	–	–	–	–	–
B3	February	6	0.23	0.041	0.086	0.051	0.022	0.72	0.008
A1	February	7	6	0.250	–	–	–	–	–
A1	April	8.5	3	0.180	–	–	–	–	–
B3	May	10	0.14	0.007	0.018	0.049	<0.005	0.19	–
A1	June	16	1.00	0.120	–	–	–	–	–
B3	March	17	0.20	0.041	0.056	0.038	0.016	0.37	–
A1	October	20	–	0.092	–	–	–	–	–
B3	June	26	0.13	0.039	0.088	0.058	0.031	0.55	0.031
C3	January	26	1.00	0.040	0.017	0.670	0.024	–	0.012
B3	July	27	0.22	0.058	0.100	0.069	0.027	0.67	0.009
A1	July	28	–	0.130	0.600	0.059	0.009	–	0.010
B3	October	33	0.21	0.034	0.038	0.070	0.027	0.25	0.009
B3	April	35	0.29	0.042	0.068	0.053	–	2.34	–
C2	July	35	–	–	–	–	0.027	–	–
A1	August	41	–	0.120	–	–	–	–	–
C1	January	45	8.00	0.150	0.093	6.410	0.21	–	0.340
B2	November	46	0.12	0.025	0.019	0.010	0.006	3.29	0.010
B3	August	47	0.22	0.060	0.100	0.064	0.025	0.61	0.007
B3	September	47	0.60	0067	0.096	0.059	0.017	0.60	0.009
B1	November	48	0.23	0.056	0.061	0.025	0.026	0.93	0.015
B1	June	51	0.13	0.041	0.095	0.063	0.030	0.58	0.011
C1	May	53	–	–	0.066	0.063	<0.005	3.58	–
B2	January	58	0.10	0.012	0.011	0.017	0.006	1.38	–
C3	May	59	–	–	–	0.024	<0.005	1.69	–
C1	August	64	–	0.170	0.082	0.049	0.010	2.32	–
B2	February	70	0.10	–	0.013	0.008	–	0.34	–
B1	August	76	0.24	0.069	0.120	0.073	0.026	0.70	0.008
B1	July	81	0.25	0.062	0.110	0.068	0.028	0.70	0.009
B1	September	81	0.60	0.094	0.110	0.067	0.025	0.68	0.010
B2	June	81	–	–	0.018	0.023	0.012	1.06	–
B2	July	81	–	0.015	0.023	0.022	0.007	0.70	–
B2	August	81	0.06	0.018	0.026	–	0.007	0.31	–
B2	October	82	0.08	0.009	0.008	0.032	–	0.93	0.005
B2	September	83	1.00	0.017	0.020	0.049	0.005	0.73	0.011
B1	February	94	0.24	0.045	0.093	0.056	0.023	0.76	0.009
A1	January	100	2.00	0.130	–	–	–	–	–
B1	October	100	0.26	0.044	0.045	0.076	0.031	0.26	0.006

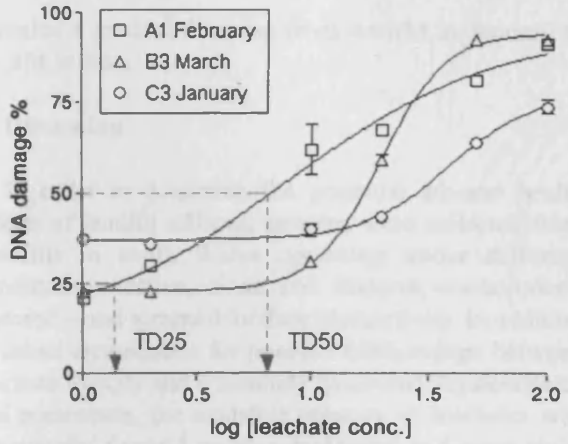

Fig. 4. Plasmid DNA damage by undiluted south Wales landfill leachates illustrating extrapolation of TD25 and TD50 values.

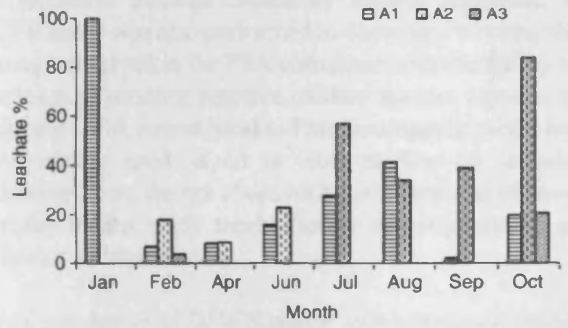

Fig. 5. Plasmid scission assay TD50 values for landfill A wells A1, A2 and A3. Grey boxes indicate TD25 obtained (Jul, Aug, Sep, Oct).

3.3.1. Landfill A (active and contained site)

The June and September samples collected from collective sump A1 produced the highest equivalent H_2O_2 compared to all other landfill A samples analysed. The other A1 leachates yielded between 51 and 85 μM equivalent H_2O_2, with overall landfill A leachates

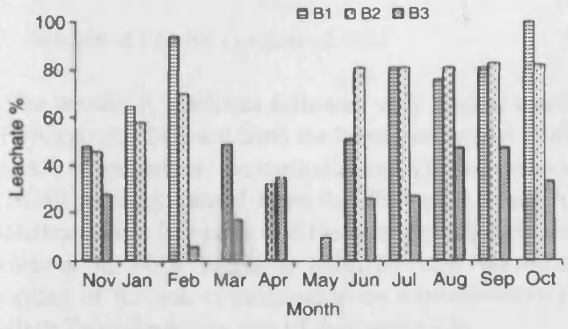

Fig. 6. Plasmid scission assay TD50 values for landfill B wells B1, B2 and B3. Grey boxes indicate TD25 obtained (Mar, Apr).

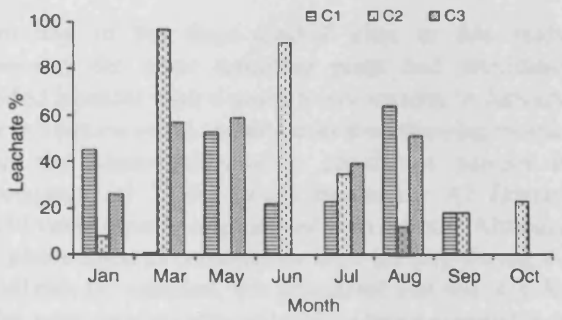

Fig. 7. Plasmid scission assay TD50 values for landfill C wells C1, C2 and C3. Grey box indicates TD25 obtained (Aug).

yielding between 45 and 290 μM equivalent H_2O_2. The A2 leachate from the restored phase produced its highest equivalent H_2O_2 in April, when it was at its most toxic according to the PSA.

3.3.2. Landfill B (dilute and disperse site)

Landfill B leachates B1 and B2 appear to exhibit similar ranges of equivalent H_2O_2, with levels existing below 60 μM at all time points assessed. In contrast to these two leachates, samples from the leachate reservoir B3 display highly elevated levels of equivalent H_2O_2, with levels reaching approximately 300 μM equivalent H_2O_2 in February and May.

3.3.3. Landfill C (restored and contained site)

Leachates collected from the collective sump C1 exhibited a steady rise in levels of equivalent H_2O_2 generated from 43 μM equivalent H_2O_2 in January to 86 μM equivalent H_2O_2 in September. The January and September leachates collected from C2 produced the highest fluorescence intensity compared to the other landfill C leachates analysed by the DCFH assay, with levels reaching approximately 150 μM equivalent H_2O_2; whilst leachate collected from the same well in July exhibited a lower response (72 μM equivalent H_2O_2). Detected equivalent H_2O_2 levels in C3 leachates

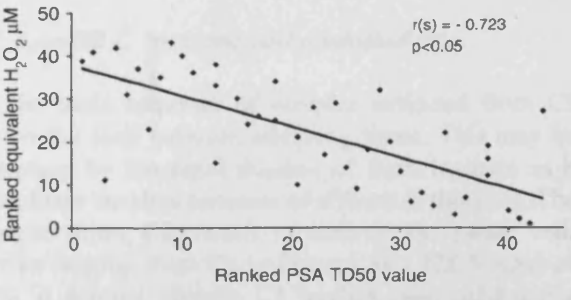

Fig. 8. Spearman's rank correlation of PSA toxicity versus equivalent H_2O_2 μM of leachates from various south Wales landfills.

revealed a gradual decrease from 44 μM in January to 10 μM in May.

4. Discussion

In order to determine the potential adverse health effects of landfill effluent, samples were collected from landfills in south Wales operating under different conditions – active, dilute and disperse, containment, restored – and screened for their bioreactivity. In addition to initial examination for possible relationships between leachate toxicity and commonly measured physicochemical parameters, the oxidative capacity of leachates was specifically focused on as a biological end-point since oxidative stress and subsequent damage leads to inflammogenic and carcinogenic health effects. A well-established, sensitive plasmid scission assay was used to detect the oxidative damage caused by landfill leachates. A DCFH assay was also performed to determine whether the damage observed in the PSA correlated with the ability of leachates to produce reactive oxidant species capable of inducing DNA strand breaks. This fluorogenic probe has been widely used as an *in vitro* marker for cellular oxidative stress; the novel use with environmental effluent samples in this study merits further investigation as an indicator of bioreactivity.

4.1. Correlation of DNA damage with physicochemical parameters

Although no significant correlation between toxicity measured by the PSA and commonly measured effluent determinants was detected, a significant association between the concentration of MCPP and TD50 values was observed after ranking analysis ($r_s = -0.817$, $p < 0.05$). Classed as a product of pesticide degradation, MCPP has been found in many MSW leachates and is often used as a tracer in studies of groundwater compromised by leachate pollution (Kjeldsen et al., 2002).

4.2. Landfill A (active contained site)

The landfill A leachates followed very similar trends in PSA toxicity. Effluent from the lined, active part of the site, A3, is drained into the central sump A1. Comparison of EC50 values obtained from the PSA of A1 and A3 leachates during February and October revealed similar toxicities at 50% leachate dilution and therefore sampling of A1 was considered to be representative of leachate from the active site of this waste site.

Effluent from A1 collected in February and April were two of the most toxic leachate samples collected from any of the three landfill sites in this study. However, the same sampling point had previously yielded leachate with a much lower toxicity in January. The evaluation of A1 toxicity over the following months with the observation of a consistent pattern in subsequent A1 TD50 values means the A1 January TD50 value must be approached with caution. Although A2 also exhibited consistently high toxicity during the initial months sampled, it is postulated that this was due to the temporary practice of leachate being pumped from the active phase to the restored phase whilst the site operators were awaiting effluent discharge consent from the regulatory authorities (personal communication, Hutchings, 2006). Further sampling of A2 during the remainder of the study appeared to corroborate this theory. The effluent collected from this section of the landfill displayed very weak toxicity as indicated by generation of TD25 values in lieu of TD50 values.

4.3. Landfill B (dilute and disperse site)

Landfill B displayed the most distinct well-to-well trends in toxicity, with the greatest toxicity exhibited by the leachate reservoir, B3. Although pipe B1 was originally installed to collect surface water, samples collected from it were nearly as toxic as freshly generated leachate collected from B2, the pipeline collecting effluent from the waste mass. It appears that the toxicity of the effluent from B1 and B2 becomes amplified once in the leachate reservoir B3. Landfill B effluent collects in B3 before being released to the public sewer system. The reactivity of landfill B leachates in the DCFH assay display two distinct responses — a lower range of equivalent H_2O_2 concentrations elicited by both B1 and B2 leachates, whilst the other response was elicited by B3 leachate which generated a higher range of equivalent H_2O_2. These two distinct groups of responses were reflected in results of the PSA, where leachates B1 and B2 appeared to follow similar trends.

4.4. Landfill C (restored and contained site)

The toxic response of samples collected from C3 varied the least between sampling times. This may be explained by the rapid dilution of fresh leachate as it mixed into the slow turnover of effluent in this pool. The samples from C2 varied in toxicity the most, with TD50s ranging from 8% in January to a TD25 value of 12% in August. Sample C1 leachate also exhibited a fluctuation in toxic response with TD50s ranging from 1% in March to 64% in August.

It can be concluded that leachate collected from the total waste via sump C1 maintained a higher degree of toxicity than C2 leachate. The greater variance seen in the C2 samples may be due to the conditions within the waste mass proximal to the catchment area of C2 whilst C1 was derived from the total waste mass, which may show less variation in composition than specific areas within the landfill. It is also noted that C2 leachate was at its least toxic when C1 leachate was also at its least toxic.

4.5. Correlation of DNA damage with reactive oxidant species

Detected equivalents of H_2O_2 were used to indicate the presence of reactive oxidant species within the leachates. In order to determine whether the indirect assessment of H_2O_2 generation was linked to the toxicity measured by the PSA, a comparison between the PSA TD50 values and equivalents of H_2O_2 was conducted by Spearman's rank correlation. This showed a significant correlation was obtained with $r_s = -0.723$ ($p < 0.05$) (Fig. 8).

5. Conclusions

Although seasonal bioreactivity data is presented here, assessment of the seasonal variation of leachate toxicity, as measured by PSA and DCFH assays, does not reveal an easily elucidated trend associated with most commonly measured physical parameters such as BOD, COD, SS, pH and metals in this study.

According to the PSA, the active and largely uncapped phase of landfill A produces some of the most DNA-damaging leachates whilst the freshly generated leachate from the unlined and partially capped landfill B are some of the least toxic effluent analysed. However, the leachate released directly from landfill B leachate storage reservoir into the public sewer system reveals a consistently amplified toxicity. Leachate collected from the closed (capped), contained landfill C displays highly variable toxicity profiles but these results indicate that although the volume of leachate discharged from landfill C is approximately 20% of landfill A, fresh landfill C leachate can be as toxic as leachate generated by landfill A; a currently operating site. The novel use of the oxidant-sensitive probe DCFH in this study suggests there may be a moderate positive association between the amount of DNA damage and the generation of reactive species. Current environmental risk assessments regarding waste effluents are based on measurements of physicochemical parameters; however, this does not take into account the interaction of complex chemical mixtures with biological systems. The PSA and DCFH assay have shown a possible link between MSW leachates and generation of ROS that must be further investigated with characterisation of groundwaters proximal to the landfill sites of interest.

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Bioreactivity of municipal solid waste landfill leachates—Hormesis and DNA damage

Lata Koshy[a,*], *Tim Jones*[b], *Kelly BéruBé*[a]

[a]School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, UK
[b]School of Earth, Ocean and Planetary Sciences, Cardiff University, Museum Avenue, Cardiff CF10 3YE, UK

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ABSTRACT

The issue of domestic waste is recognised as one of the most serious environmental problems facing the nation. With the UK producing 35 million tonnes of municipal solid waste per annum, an understanding of the ranges of toxicity of landfill emissions is crucial to determine the degree of concern we should have about the potential effects these waste sites could have upon nearby populations and the surrounding environment. The aim of this study was to evaluate the bioreactivity of landfill leachates in terms of their capacity to damage ROS-sensitive bacteriophage plasmid DNA and induce toxicity in a commercial photobacterium toxicity assay, based on the light emission of *Vibrio fischeri* bacteria (ROTAS™). The bacterial assay revealed widespread biostimulation and a hormesis response in the bacteria, with α-, β- and γ-response curves observed following exposure to the different landfill leachates. Different biological mechanisms lead to variations in bioreactivity, as seen in the plasmid DNA scission and ROTAS assays.

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1. Introduction

As an unavoidable issue, municipal solid waste (MSW) landfills are of great environmental concern. The UK disposes over 35 million tonnes of waste into municipal landfills each year, largely derived from households (Defra, 2006), which results in a wide range of by-products in the form of leachate, gas and particulate emissions, although the latter is only released from active, open landfills (Defra, 2004). Landfill gas is occasionally collected as a condensate due to the moisture inherent within landfills and perforated pipes collect leachate prior to release or water treatment. Modern landfills are constructed to contain as much of these emissions as technology permits, but it has been accepted that all landfills will leak to some extent, causing varying levels of environmental pollution (McQuade and Needham, 1999).

Landfill leachate is a complex effluent of waste breakdown products and particulates, formed by the movement of rainwater and moisture through the waste mass. In sites which operate under containment principles, leachate migrates to the lowest point within the waste mass and is drained away by a series of pipes into a central sump or lagoon. Current licences for discharge consent of UK landfill effluents are based on the physicochemical determinants such as biological oxygen demand, pH, chemical oxygen demand and total dissolved solids (TDS) (Defra, 2002). However, these parameters do not necessarily indicate the toxic potential of the leachate. Previous ecotoxicological studies have resulted in detrimental effects being observed in murine, invertebrate, aquatic and microbial species (Schrab et al., 1993; Baun et al., 2004; Silva et al., 2004; Li et al., 2006). However, extrapolation of these model systems to the human population requires multidisciplinary consideration.

The objective of this current research is to (1) evaluate a 'whole-organism' toxic response of *Vibrio fischeri* and (2) determine if it is comparable to the bioreactivity revealed by an *in vitro* plasmid scission assay (PSA) when exposed to leachates from different types of MSW landfills: active and containment, dilute and disperse and containment (recently restored) landfills, all located in Wales, UK. Bioreactivity was identified by the *in vitro*

*Corresponding author. Tel.: +44 2920 876810; fax: +44 2920 875211.
E-mail address: KoshyL@cardiff.ac.uk (L. Koshy).
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PSA, whilst whole-body toxicity was assessed by *V. fischeri*, using the commercially available ROTAS™ system. The PSA has been recently used in a previous investigation to assess the ability of landfill leachate to cause DNA damage to a ROS-sensitive plasmid (Koshy et al., 2007). Toxicity tests with *V. fischeri* in commercially available kits such as the ROTAS, ToxAlert® and Microtox® systems have been used extensively in environmental monitoring of contaminated soils and aquatic systems, leading to their regular use by national environmental agencies (US EPA, 1993; Gustavson et al., 1998).

2. Material and methods

2.1. *Landfill sampling sites and leachate collection methodology*

The waste disposal sites of interest all lie within a 25 km radius from the city of Cardiff in Wales, UK (Fig. 1), and have been selected due to their different characteristics. The sites have been deemed representative of large, modern landfills, where past practices have meant that hazardous wastes have been buried within them. Landfills A and B are currently accepting MSW whilst landfill C is closed to waste tipping. Landfill A is a containment landraise site, whilst landfill B is a dilute and disperse landfill site. Both A and B have accepted hazardous waste in the past including motor vehicle parts, tyres and clinical waste; these areas have since been capped. Landfill B was originally used for industrial waste from the steel industry before its use for MSW. Landfill A is located in the city of Cardiff whilst landfill B is situated in a rural south Wales valley. Landfill C is a fully restored containment site built in a depression high on the side of a rural south Wales mountain. All landfill leachates were collected by grab-sampling from the collective sump, restored phases, active phase and leachate reservoir within a week spanning January and February 2007. A sample of groundwater collected from an up-gradient aquifer proximal to landfill C was acquired as a model of a clean environmental sample to be analysed along with the effluent samples in the bioassays of interest (Table 1).

All leachate samples were collected in 250 mL HDPE bottles with minimal headspace and filtered through mixed cellulose ester 0.45 μm membranes (Fisher Scientific, UK) in the laboratory before being stored at 4 °C in the dark until use.

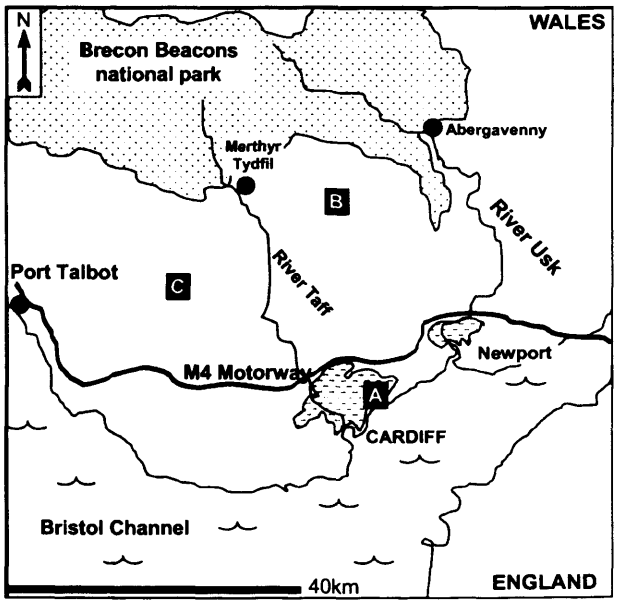

Fig. 1 – Landfill sites of interest in south Wales, UK. A = active contained site; B = active, dilute and disperse site; C = restored and contained site (adapted from Koshy et al., 2007).

2.2. *Analytical techniques*

2.2.1. *Physicochemical analysis*

Parameters measured at the time of sampling were pH and redox potential (HANNA Instruments, Meter HI-991002), conductivity and TDS (HANNA Instruments, Meter HI-98311). Ion chromatography for Cl^- and SO_4^{2-} was performed at Cardiff University (Dionex, Model DX-80), whilst data for Ammoniacal-nitrogen (NH_4^+-N) were provided by the landfill site operators following analysis by contracted and UKAS-certified laboratories.

2.2.2. *Plasmid scission assay*

The plasmid ΦX174 RF DNA molecule (Promega, London, UK) was used in the PSA. Filtered leachate samples were diluted in molecular grade water (Sigma-Aldrich, UK) and incubated with 200 ng ΦX174 RF DNA in a final volume of 20 μL ($n = 4$).

Table 1 – Sampling well characteristics of landfill sites of interest

Site	Type	Operation since	Waste type (in addition to MSW)	Collective sump	Restored phase	Leachate reservoir	Groundwater
A	Active; contained	1978–present	Mixed industrial, chemical, bulky	A1	A2	–	–
B	Active; dilute and disperse	1981–present	Iron and steel industrial	B1	B2	B3	–
C	Restored; contained	1988–2002	Industrial, filter cake	C1	C2	C3	C4

A negative control was established using incubations of DNA in molecular grade water and positive control consisted of restriction enzyme Pst I (Promega, London, UK) digest. Samples were gently agitated for 6 h before 3.33 μl loading dye (Promega, London, UK) was added. Samples were electrophoresed on a 0.6% agarose 0.25% ethidium bromide gel for 16 h at 30 V in 1× Tris–borate–EDTA buffer (Fig. 2). Electrophoresed gels were imaged using VisionWorks® software (Ultraviolet Products Ltd., UK) and densitometric analysis was performed with Genetools® software (Syngene® Systems, UK).

The relative amount of damaged DNA (relaxed open circle form plus linearised form) in each lane was calculated as a percentage of the supercoiled intact DNA. Gels with less than 10% damage in the water controls were accepted for analysis. The data were fitted on a non-linear regression sigmoidal model using GraphPad Prism 2 (GraphPad Software Inc., CA, USA). The concentration of leachate to produce 25% and 50% damage (TD25 and TD50) was determined in order to facilitate comparison between samples.

2.2.3. *ROTAS assay*

The ROTAS system was purchased from Cybersense Biosystems (Oxfordshire, UK). The original ROTAS system is designed to reflect the health of the bacterial population, indicated by light emission after 15 min exposure to waste effluent. Light emission is a function of the respiratory activity and indicative of the metabolic rate of the organisms. Although other commercial acute toxicity tests with *V. fischeri* are often limited to 15–30 min duration, the investigators in this study discovered that this incubation period was not feasible, as late-onset adverse effects were masked by biostimulation. Inspection of the luminescence data revealed an early-phase decrease in light production in several samples followed by massive biostimulation. This would ordinarily have been reported as a non-toxic leachate sample. Hence, the initial duration of the ROTAS system as a 15 min acute toxicity test was extended to 100 min to detect sub-acute toxicity of various MSW landfill leachates. This action was supported by the current literature which reports significant differences in bacterial viability when exposed to adverse conditions for increasing incubation times (Fulladosa et al., 2005).

The lyophilised organisms were rehydrated in 2% NaCl for 50 min, following which 1 mL aliquots were transferred into a 24-well plate in a Cysense luminometer (Cybersense Biosystems). The system was calibrated to establish basal levels of bacterial luminescence prior to exposure with 1 mL undiluted leachate sample. Readings were collected every minute over a 100 min period. Simultaneous incubations of negative controls were incubated with 2% NaCl and positive control wells were exposed to 10 mg/L $CuSO_4$ (Fisher Scientific, UK). The leachates were between pH 6 and 8 for analysis ($n = 3$), adjusted with 0.1 N HCl if necessary. Sample bioluminescence was calculated as a proportion of the saline control.

3. Results

3.1. *Physicochemical analysis*

The physicochemical parameters of each sample are presented in Table 2. Leachates A1 and C2 had the highest conductivity levels of 10,356 and 15,648 μS/cm, respectively. These samples were also the most reducing and had the highest levels of colloidal inorganic and organic content, indicated by the highest TDS values seen in this study. In contrast, the groundwater sample, C4, had the lowest conductivity and TDS levels recorded (169 μS/cm and 84 mg/L, respectively). Landfill B leachates exhibited little intra-site variation in physicochemical characteristics except for B2 (restored phase), which contained the least amount of SO_4^{2-}. The pH values for all sites ranged from 7.1 to 8.3. SO_4^{2-} levels ranged from 3 to 270 mg/L in all samples, except leachate C2, which contained 4166 mg/L SO_4^{2-}.

3.2. *Plasmid scission assay*

Not all PSA dose–response profiles for the leachates are presented, as trends for all samples were found to be similar; however, an example has been provided (Fig. 3, leachate A1). A non-linear dose–response model was established in order to compare PSA results for all leachates. The majority of samples were assigned TD50 values, but a TD25 value was generated when maximum sample toxicity fell below 50% (Table 2). Leachate A1 produced the most DNA-damaging leachate, with a TD50 of 10%. Effluent collected from B2 was the least bioreactive leachate according to the PSA, with a TD25 of 100%. Landfill B leachates were generally less DNA-damaging than leachates collected from landfill C. The

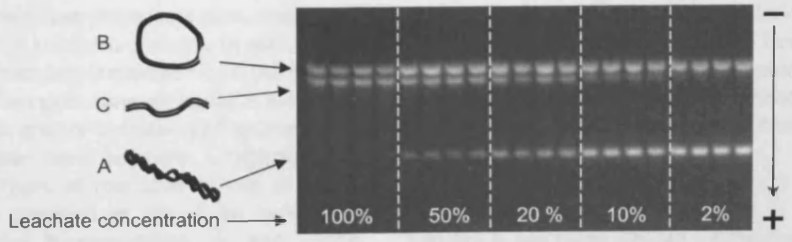

Fig. 2 – Plasmid scission assay of ΦX174 RF DNA incubation with various concentrations of landfill leachate. Conformations at varying levels of progressive damage; A: undamaged supercoil, B: preliminary damaged relaxed coil, C: severely damaged linearised DNA (adapted from Koshy et al., 2007).

Table 2 – Bioreactivity and physicochemical characteristics of leachates

Leachate	ROTAS response	PSA TD25 (%)	PSA TD50 (%)	NH_4^+-N (mg/L)	Temp (°C)	Cl^- (mg/L)	SO_4^{2-} (mg/L)	pH	TDS (mg/L)	Redox potential (mV)	Conductivity (μS/cm)
A1	α	–	10	479	22	920	3	7.3	5196	−151	10,356
A2	β	–	77	<0.3	10	3	3	7.2	822	48	1638
B1	γ	75	–	153	22	179	202	8.1	3172	53	6360
B2	γ	100	–	191	22	226	92	7.7	2596	74	5200
B3	γ	–	85	167	22	298	270	8.0	2876	66	5656
C1	γ	–	57	780	20	1048	65	7.7	3856	10	7756
C2	α	20	–	–	26	1194	4166	7.2	7736	−391	15,648
C3	β	–	75	28	6	436	108	8.3	1957	−14	3974
C4	β	–[a]	–	–	8	13	27	7.5	84	16	169

[a] Sample C4 exhibited less than 25% DNA damage in the PSA at any dilution tested, and was classed as a non-toxic sample.

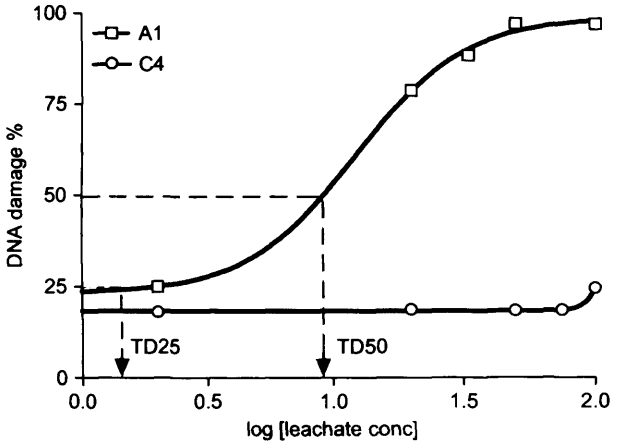

Fig. 3 – Plasmid DNA damage by undiluted south Wales landfill leachates illustrating extrapolation of TD25 and TD50 values.

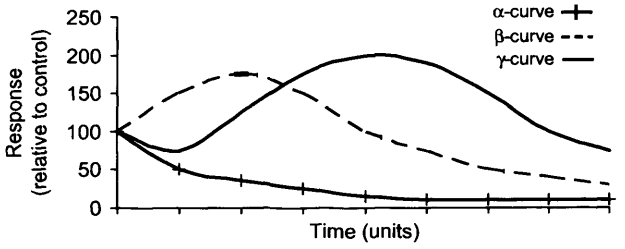

Fig. 4 – ROTAS assay profiles depicting α-, β- and γ-response profiles.

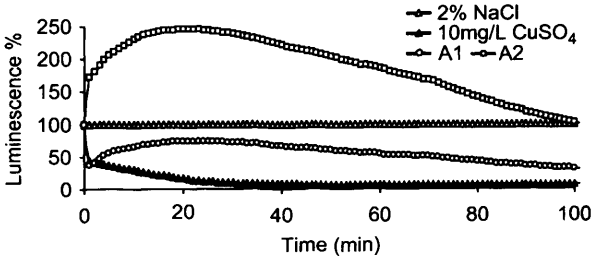

Fig. 5 – ROTAS assay response profiles of landfill A leachates.

landfill C groundwater sample (C4) exhibited the lowest levels of DNA damage and did not fit the non-linear model of the leachate samples. Extrapolation of TD25 proved impossible, and C4 was hence classed as a non-bioreactive sample.

3.3. ROTAS assay

The sensitivity of *V. fischeri* test organisms was confirmed with the inclusion of Cu^{2+} in the form of $CuSO_4$ in each assay; this also verified the correct implementation of the ROTAS protocol. The majority of samples from all landfills exhibited a stimulatory effect, with greatly elevated bioluminescence observed in seven of the nine leachate incubations. A schematic of the three types of response curves obtained by the ROTAS assay is provided in Fig. 4 for reference. Of the samples exhibiting biostimulation, β- and γ-type hormesis response curves were obtained, with the response ranging from 130% to 245% compared to the saline controls (Figs. 5–7).

3.3.1. *ROTAS response—landfill A*

Landfill A active-phase leachate A1 produced an atypical α-curve (Fig. 5). Although bacterial luminescence decreased to 37% within 1 min post-exposure, this was followed by a short recovery period peaking at 25 min, before a permanent fall in light output was observed. It was noted that this transient recovery did not attain pre-exposure luminescence levels during the episode. The restored-phase leachate A2 produced a β-response, with luminescence decreasing to sub-control levels at 100 min.

3.3.2. *ROTAS response—landfill B*

Landfill B leachates yielded γ-type curves, with all samples undergoing an immediate fall in luminescence within the 1st minute post-exposure (Fig. 6). This was followed by biostimulation above control luminescence levels, peaking by

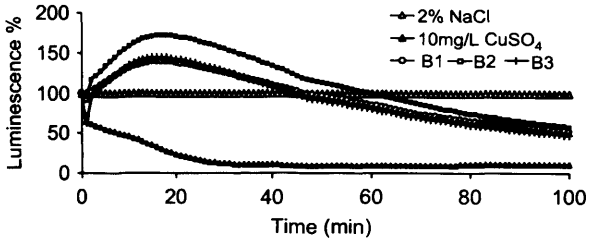

Fig. 6 – ROTAS assay response profiles of landfill B leachates.

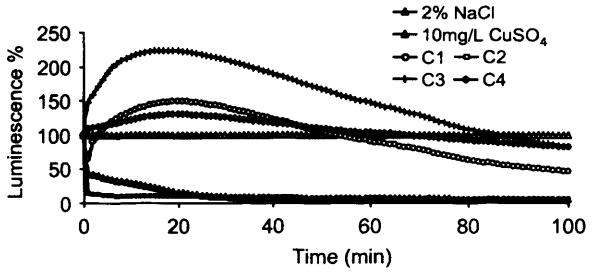

Fig. 7 – ROTAS assay response profiles of landfill C leachates.

20 min. All leachates then produced a permanent fall in light production; B1 and B3 fell below control level at approximately the same time (44–45 min post-exposure) while leachate B2 had a more protracted response, falling to sub-control values at 60 min. Although effluents B1 and B3 shared very similar ROTAS biostimulation profiles, sample B3 exhibited the greatest immediate decline in light production, falling to 63% before luminescence increased to levels comparable to that of leachate B1. Leachate B1 and B2 had similar initial effects upon the bacteria, causing 91% and 95% luminescence within the 1st minute post-exposure.

3.3.3. ROTAS *response—landfill C*

Landfill C samples produced the most varied ROTAS responses, with α-, β- and γ-curves obtained (Fig. 7). Collective sump leachate C1 produced a γ-curve, with luminescence falling to 65% within the 1st minute post-exposure. This was followed by biostimulation, peaking at 149% after 20 min, before a permanent decrease in luminescence, with light production falling below control levels after 53 min incubation time. Active-phase leachate C2 yielded a typical example of a steep α-type response curve, where luminescence fell to 14% after 1 min and remained below this level for the duration of the assay. Samples C3 and C4 shared similar ROTAS profiles; effluent from the reservoir C3 produced a β-curve, peaking at 245%, which was more pronounced than that of leachate C4, which peaked at 130%. Effluent C3 also exhibited a prolonged decline in light production compared to C4, with sub-control luminescence thresholds being crossed at 86 and 65 min, respectively.

4. Discussion

An integrated chemical and biological assessment strategy of MSW landfill leachates is the preferred method to determine the hazard such waste effluent may pose to the environment and surrounding populations (Isidori et al., 2003). In order to find a correlation between two bioassays that could be used in future screening approaches to pollution assessment, various leachate effluents from three different types of MSW landfills were sampled and assessed in early 2007. These samples were analysed for target physicochemical parameters and assessed by a commercial luminescent bacteria (*V. fischeri*; ROTAS) toxicity assay and a plasmid DNA scission assay (PSA) as indicators of toxicity and bioreactivity.

The unexpected ROTAS biostimulation response ranged from 130% for groundwater C4 to 245% for leachate A2. Although the groundwater sample (C4) was collected as an environmental clean water sample, and had a negligible effect upon DNA damage in the PSA, the mild biostimulation response reported in the ROTAS system is of interest since it has a bearing on the interpretation of the waste effluent effects upon the *V. fischeri*.

The observed ROTAS biostimulation could be due to the phenomenon of hormesis, characterised by a biphasic response. Hormesis has been the subject of a resurgence in interest, with recent reviews by Calabrese and Baldwin (2002) defining hormesis as a "dose–response phenomenon characterised by a low dose stimulation and a high dose-inhibition", while also being defined as an "adaptive response" of the organism to the effects of toxicant exposure (Stebbing, 2000). This result is not commonly discussed in ecotoxicological monitoring studies, as the end-points emphasised are usually lethal or inhibitory effects. Nevertheless, this type of response has been previously reported by other investigators using *V. fischeri* (Christofi et al., 2002; Davoren et al., 2005).

Three types of response curves were identified in the ROTAS system. The α-curve is the typically reported dose–response toxicological profile in which hormesis is not a feature. Here, inhibition is seen in the absence of any stimulatory effect. The β and γ profiles have been described as biphasic, where both stimulation and inhibition are evident. The β-profile is characterised by a single, direct stimulation peak, whilst the γ-curve is indicative of an over-compensation effect (Calabrese and Baldwin, 2002). In the latter, an initial inhibition is quickly followed by stimulation and an eventual inhibitory response.

The mechanism(s) of toxicity underlying the biostimulation of the microorganisms is difficult to interpret based upon the physicochemical parameters collected. Landfill leachates are a highly chemical complex mix of organic and inorganic compounds. Such mixtures may act in synergy to cause toxicity (Tsiridis et al., 2006). In this study, high levels of TDS, conductivity and redox potential have been the closest indicators of bacterial toxicity, with leachates from the active phases of landfill A (A1) and C (C2) causing α-type responses in the ROTAS assay, with no hyperstimulation observed. However, these samples elicited very different levels of damage in the PSA, with C2 being significantly less bioreactive than A1 (TD25 of 20% and TD50 of 10%, respectively).

It was not possible to determine which of these components (redox, TDS or conductivity) was the dominant stressor, but it is likely that a combination of all three would have played a part in whole effluent toxicity of A1 and C2 in the

ROTAS assay. The highly reducing conditions of leachates A1 and C2 may have caused a deleterious effect upon the aerobic metabolism of the bacteria, inhibiting respiration, and hence bioluminescence. TDS and conductivity are indicators of the ionic strength of the effluent and aquatic toxicity of highly ionic effluents have been shown in various species (Chapman et al., 2000; Kennedy et al., 2005). Effluents that exhibit extremes of TDS and conductivity, as is seen in leachates A1 and C2, may induce ionic imbalance in the exposed test organism, causing osmotic stress and leading to mortality. Goodfellow et al. (2000) suggested that effluents with TDS above 1340 mg/L (and conductivity above 2000 μS/cm) may cause toxicity in freshwater species. In the case of *V. fischeri*, a marine organism, this threshold may be expected to be higher due to the natural environment the bacteria colonise. In this study, samples with a TDS value of approximately 4000 mg/L and conductivity of approximately 8000 μS/cm did not exhibit mortality.

In the three samples that produced β-responses, one of these was the groundwater (C4) sample. The other two samples were collected from the restored phase in landfill A (A2) and the leachate reservoir in landfill C (C3), which had similar results in the PSA (TD50s of 77% and 75%, respectively). These displayed an immediate over-compensation stimulation, which manifested as hyper-production of light from the microorganisms without the initial decrease as seen within 1 min post-exposure to leachates A1 and C2. As time progressed, the levels of luminescence fell below those of control values.

Of the four leachates, which displayed γ-responses, it is interesting to note that all landfill B effluent samples fell into this category, as did leachate from the collective sump of landfill C (C1). In this type of response, there was an initial α-response, which was overcome after the 1st minute post-incubation, resulting in a β-response. Physicochemical parameters of landfill B leachates were very similar, with C1 values of TDS and conductivity in a comparable range.

In all cases of leachate hyperstimulation, the observed slope of decreasing luminescence after the microorganisms reached their maximum light production was very similar; therefore, the time at which the *V. fischeri* returned to control levels was dependent on the extent of biostimulation attained. In all cases, this was within 25 min post-exposure to leachate. Groundwater sample C4 may be causing bioluminescence via a different mechanism to that of the leachates, resulting in a different ROTAS profile.

5. Conclusion

This investigation into MSW leachate bioreactivity, as measured by an *in vitro*, plasmid DNA scission assay, and leachate toxicity gauged by a whole-organism bacterial luminescence assay (ROTAS), found little correlation between the two systems. The mechanisms underlying the two bioassays are likely to be varied and complex, as indicated by the concomitant lack of association with commonly measured physicochemical parameters. The leachates generally elicited temporal-dependent hormetic responses in the ROTAS system, whilst the PSA system indicated a more commonly reported sigmoidal dose-dependent response.

Leachates from currently active MSW landfills display varying levels of bioreactivity and toxicity. Currently operating landfills A and B displayed closest correlation between bioreactivity and toxicity for all leachates collected. The closed, inoperative landfill C displayed intra-site-specific variation, with all analysed effluents displaying different toxic responses in the ROTAS and PSA systems.

Environmental monitoring kits have the benefit of being relatively rapid and cost-effective. Nevertheless, the data presented here also exemplify the caution that must be exercised when unpredicted results are obtained as toxicity may be masked by pre-programmed test duration.

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REVIEW ARTICLE

Characterization and bioreactivity of respirable airborne particles from a municipal landfill

Lata Koshy[1], Timothy Jones[2], and Kelly BéruBé[1]

[1]School of Biosciences, Cardiff University, Museum Avenue, Cardiff, UK, and [2]School of Earth and Ocean Sciences, Cardiff University, Park Place, Cardiff, UK

Abstract

With an increasing population and greater pressure on land-use, the possible problems of landfilling are of increasing concern. These concerns include the possible adverse health effects arising from living in the vicinity of municipal solid waste (MSW) landfills. Human exposure to potential landfill emissions by respiratory, gastrointestinal or dermal mechanisms warrants further investigation. PM_{10} and $PM_{2.5}$ from a UK landfill were physicochemically characterized and their bioreactivity screened by a plasmid scission assay in comparison with an urban PM collection. Preliminary data from human toxicology pathway-specific microarrays indicate landfill PM_{10} presents a comparable geobiological insult to urban PM_{10} in a human tracheobronchial tissue model.

Keywords: *Landfill; PM_{10}; toxicity; bioreactivity; plasmid scission assay*

Introduction

A recent study by Elliot et al. (2001) estimated that 80% of the UK population reside within 1.5 miles of a working or closed landfill site. The major municipal solid waste (MSW) landfill emissions from an operating site are particulate matter, leachate and landfill gas. Although there are many sources of literature available on landfill leachate and gas characterization, this mini-review will focus on the less reported field of ambient airborne particles related to landfills. Particulate matter (PM) with aerodynamic diameters of less than 10 or 2.5 μm (PM_{10} and $PM_{2.5}$) is recognized as an important cause of adverse human health (Englert 2004, Kappos et al. 2004, Brunekreef & Forsberg 2005). There is a current paucity of information on landfill PM, meaning conclusions about landfill PM require further research (Defra 2004).

Landfill dust is generated by a variety of mechanical and chemical processes. These include: the movement of heavy dustcarts and site vehicles over dry unpaved access roads and previously deposited waste; diesel exhaust fumes and brake emissions from on-site vehicles; action of tipping waste at the working face raises plumes of dust, notably on elevated ground, which are exposed to windy conditions; waste compaction by bulldozers and crushers; and stockpiles of bare earth required for daily waste coverage are susceptible to resuspension and dispersion by wind (Fitz & Bumiller 2000). Arid and windy conditions may lead to higher levels of entrained PM in the local atmosphere. Human exposure can occur via respiratory, dermal and ingestion pathways, and is sensitive to meteorological conditions. Vega et al. (2001) performed resuspension of surface dust from a Mexico City landfill, obtaining PM_{10} and $PM_{2.5}$ fractions for physicochemical analyses. Interestingly, their gravimetric analysis revealed a similar mass distribution to that of the UK landfill. However, their chemical analysis revealed a significantly different metal profile, with Al being the most significant metal component, whilst Fe was the most significant metal present in the UK landfill presented in this review.

In a literature-based modelling study, Macleod et al. (2006) estimated the amounts of airborne incinerator residual ash, which could be potentially released as particulates from landfills. Although these sites were not specific MSW sites in the UK, which no longer accept

Address for Correspondence: Lata Koshy, School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX, UK.

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such controlled wastes, their assessment positively identified the atmospheric dispersion of dusts as the main pathway for human exposure. The main route of exposure to landfill PM is by inhalation and possibly to a lesser extent, by ingestion. The re-dispersion of accumulated dust on clothing can increase personal exposure to PM; however this is more likely within the landfill workforce rather than public exposure (Poulsen et al. 1995).

It is important to find sensitive biological indicators of the potential harm that these emissions may cause following human exposure. Linking toxicological analysis to the geochemical data should help to determine accurately the degree of toxicity of environmental emissions produced by landfills. This study aims to determine the levels of respirable landfill particulates released from a UK MSW landfill, and to identify the acellular bioreactivity of these pollutants. We also present our preliminary data of transcriptional changes in a human epithelial tracheobronchial model upon exposure to landfill PM_{10}, and compare these observed effects to urban PM_{10} exposure.

Physicochemistry

Sample collection

The study site is a municipal solid waste landfill, located within a major UK city. Sampling at an urban location approximately 2.5 miles from the landfill was also undertaken. Two collecting systems were utilized in this study during October 2007. A Negretti selective-inlet system was used to collect PM_{10} on polycarbonate filters for characterization. The system operates at a flow-rate of 30 l min^{-1}, drawing air along a set of defined horizontal plates, through to the polycarbonate filter. Pre-calibrated settings prevent PM with a mean aerodynamic diameter of greater than 10 μm, from progressing through the elutriator (Greenwell et al. 2002). A Harvard high-volume collector, configured to collect particulates of 10–2.5 μm and 2.5–0.1 μm, was used to accumulate PM_{10} onto polyurethane foam substrates for bioreactivity assays (Jones et al. 2006).

PM characterization

Gravimetric and scanning electron microscopy (Moreno et al. 2004) analyses of landfill and urban PM revealed a similar size distribution of PM_{10} at these two locations; however, it also revealed significantly increased PM_{10} generation at the landfill compared with the urban city location, i.e. 42 and 13 μg m^{-3}, respectively. Airborne dust emissions are predominantly episodic, and highly dependent upon site operations and ambient conditions. This also means that extrapolation of gravimetric measurements to the long-term air quality indicators may not be the most appropriate use of this data (Macleod et al. 2006). Both sites generated PM_{10} with the vast majority of particles distributed within 2.5–0.1 μm aerodynamic

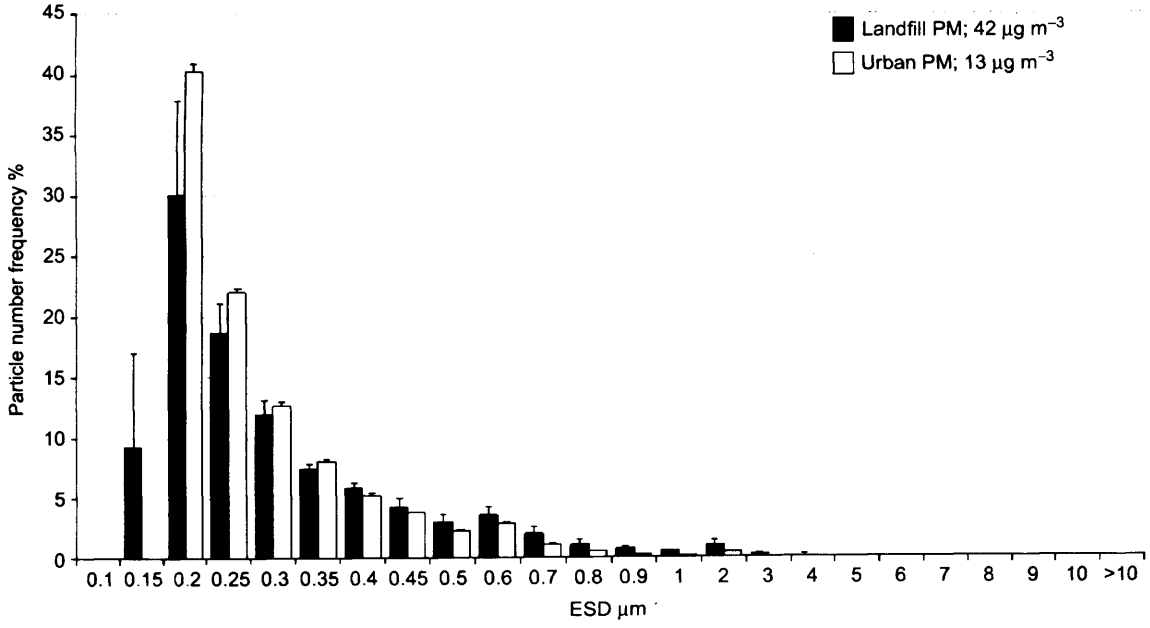

Figure 1. Skewed size distribution of PM_{10} collected from a municipal solid waste landfill and a nearby urban location, both located in the UK. The results indicate the vast majority of PM from both locations are respirable. Note the small fraction of PM 0.1-0.15 μm in the landfill collection. The landfill PM mass concentration was much higher than the urban location.

diameter (Figure 1). The dominant PM types were identified as either mineral or soot, with the dominant PM at the landfill site being mineral (98% particle number), whilst the urban PM_{10} sample comprised mostly, of anthropogenic soot PM (95% particle number).

Metal analysis

The $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ from both study sites were microwave acid-digested and analysed for total trace metals by ICP-MS (Moreno et al. 2004). The elements of toxicological concern, Fe, Zn, Ni and Pb, were prevalent in all samples. Both PM fractions, $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$, collected at the landfill contained higher overall levels of metals than the corresponding urban samples (Figure 2A, B). Landfill $PM_{10\text{-}2.5}$ was found to be significantly Fe-rich compared with the accompanying landfill $PM_{2.5\text{-}0.1}$ fraction, or the urban PM_{10} fractions (SPSS 15.0 statistics package; one-way ANOVA, $p<0.01$). The landfill $PM_{2.5\text{-}0.1}$ fraction contained significantly higher levels of Zn and Pb than the other samples ($p<0.01$).

Bioreactivity

Plasmid scission sssay

Particulates were screened by the plasmid scission assay (PSA), as an *in vitro* measurement of oxidant PM-bioreactivity upon DNA (Donaldson et al. 1997). Aliquots (200 ng) of Φ X174 RF plasmid DNA were exposed to PM_{10}, $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$. The PM_{10} sample was derived from proportional masses of $PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$. Exposure of the supercoiled DNA to damaging, oxidative PM caused a conformational change in the tertiary structure of the plasmid, to the relaxed and/or linear forms. This resultant damage was detected by gel electrophoresis and semiquantified as a proportion of the total DNA present (Koshy et al. 2007).

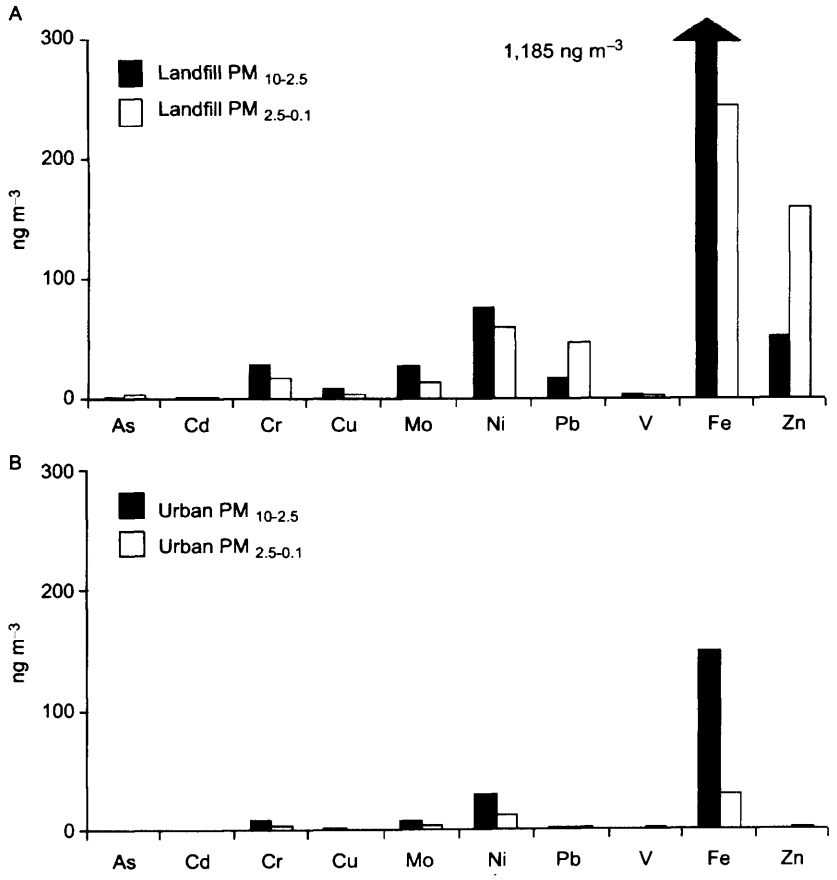

Figure 2. (A) Landfill PM; (B) urban PM. The results show that Fe is the most abundant metal in landfill and urban PM. and in the case of the landfill $PM_{10\text{-}2.5}$ fraction, this was highly significant. The other dominant metals in both size fractions of landfill PM were Zn and Pb. The urban PM contained lower metal concentrations in both fractions compared with their respective landfill samples. However, as in the landfill collection, Fe was also the most abundant metal in the urban collection.

Data were compared for statistical significance between PM sizes intrasite, and between corresponding PM sizes of the two different sites (SPSS 15.0 statistics package; two-way ANOVA and least significant difference *post-hoc* testing). The $PM_{2.5\text{-}0.1}$ fractions at both the landfill (Figure 3A) and urban sites (Figure 3B) were found to be significantly more reactive than their corresponding $PM_{10\text{-}2.5}$ or PM_{10} fractions ($p<0.01$). This finding is in agreement with other studies which report smaller sizes of PM as being more reactive (Risom et al. 2005, Diociaiuti et al. 2001). Landfill $PM_{2.5\text{-}0.1}$ exhibited a much higher oxidative capacity than the urban $PM_{2.5\text{-}0.1}$ ($p<0.05$), with the former causing 50% damage (TD_{50}) at 28 μg ml^{-1}, whilst urban $PM_{2.5\text{-}0.1}$ elicited the same level of damage at 185 μg ml^{-1}. The landfill $PM_{2.5\text{-}0.1}$ fraction was the dominant contributor to bioreactivity in the landfill PM collection. It was noted that there was no significant difference between the landfill PM_{10} and $PM_{10\text{-}2.5}$ fractions. The trend of bioreactivity of the landfill samples was in the order of $PM_{2.5\text{-}0.1} > PM_{10} = PM_{10\text{-}2.5}$.

In contrast, a positive dose-response was observed with all fractions from the urban site, in which the extent of bioreactivity was significantly dependent upon the size fraction. The trend of bioreactivity of the urban site was in the order of $PM_{2.5\text{-}0.1} > PM_{10} > PM_{10\text{-}2.5}$. All the urban size fractions, excluding $PM_{10\text{-}2.5}$, exhibited significantly higher oxidative capacity when compared with their counterparts in the landfill collection ($p<0.05$). Overall, the landfill PM_{10} bioreactivity was dominated by the less reactive $PM_{10\text{-}2.5}$ fraction, whilst the urban PM_{10} bioreactivity was dominated by the more reactive $PM_{2.5\text{-}0.1}$ fraction.

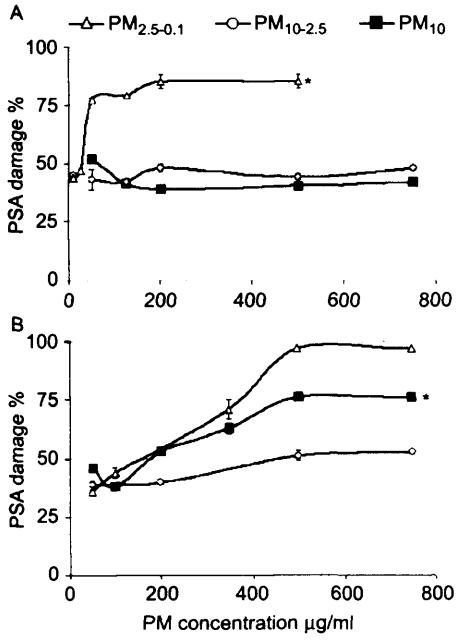

Figure 3. (A) Landfill PM; (B) urban PM. Plasmid scission assay of the different PM fractions collected at the landfill and urban locations. Data are expressed as mean ± SD ($n=3$). *Statistical significance between corresponding size fractions from the two study sites ($p<0.05$). The $PM_{2.5\text{-}0.1}$ samples from both locations were the most bioreactive, with the landfill collection exhibiting the greatest oxidative capacity.

Exposure of landfill PM to human tracheobronchial epithelial cells in vitro

The commercially available EpiAirway-100 3-D model (MatTek Corp., Ashland, MA, USA) was exposed to $PM_{10\text{-}2.5}$, $PM_{2.5\text{-}0.1}$ and PM_{10} from the landfill, and the PM_{10} fraction from the urban collection (500 μg ml^{-1}; 24h). Conventional toxicology and genomics were performed. The MTT assay was used to determine cell viability (mitochondrial activity), whilst the transepithelial electrical resistance (TEER) measurements for structural integrity were taken pre- and post-exposure. Genomic analysis of the exposed tissue was performed according to the GEArray Human Toxicology and Drug Resistance microarray protocol (SABiosciences, Frederick, MD, USA). Gene expression was determined by Significance Analysis

Table 1. Distribution of upregulated genes, grouped into gene families by percentage (landfill PM_{10} = 66 genes; urban PM_{10} = 57 genes).

Gene family	Examples of genes expressed in EpiAirway-100(≥ 1.5 fold, $p<0.05$)	Landfill PM_{10}	Urban PM_{10}
Drug metabolism	N-Acetyl transferase 2, glutathione peroxidase 1, peroxiredoxins 1and 2, glutathione S-transferase, cytochrome P450 11B2	29	28
Transcription factors and regulators	Retinoic acid receptor α, MYST histone acetyltransferase, NFKB1, v-rel reticuloendotheliosis viral oncogene homolog B	15	14
Stress response	Superoxide dismutases 1 and 2, NADPH dehydrogenase 1, paraxonase 3, nudix type 1, DNA damage-inducible transcript 3	11	9
Cell growth, proliferation and differentiation	Cyclin-dependent kinases, V-erb-b2 erythroblastic leukaemia viral homolog, macrophage migration inhibitory factor	12	7
Chaperones and heat shock proteins	Chaperonins, heat shock proteins and transcription factors, suppression of tumorigenicity 13, crystallin αA	27	28
Apoptosis	BCL-like 2, tumour necrosis factor receptor superfamily 1A, E2F transcription factor, NF-κB inhibitor α	5	5
Cell cycle	Breast cancer 2, cyclin-dependent kinases 2 and 4, DNA-damage-inducible transcript 3	2	9

of Microarrays software (Stanford University), in which changes of greater than 1.5-fold ($p<0.05$) were considered to be relevant. There were no significant adverse effects detected by TEER measurements, or by the MTT viability assay upon exposure to any of the three landfill PM fractions, or from the urban PM_{10}. However, preliminary microarray data of the landfill and urban PM_{10} indicated a significant trend of upregulation in several transcription factors and drug metabolism enzymes (Table 1).

Conclusions

PM_{10} collected at a municipal UK landfill displayed a highly skewed size distribution, with the majority of particles residing in the $PM_{2.5\text{-}0.1}$ fraction, as amorphous particulates. The study here also demonstrated that acellular ROS-induced bioreactivity of the landfill $PM_{2.5\text{-}0.1}$ fraction was shown to be significantly higher than a corresponding urban $PM_{2.5\text{-}0.1}$ sample. To our knowledge, no other publications target MSW landfill PM_{10} toxicity, and this preliminary data should be reported.

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