TOXICOLOGICAL ANALYSIS OF HOUSE DUST COLLECTED FROM SELECTED DURBAN RESIDENTIAL BUILDINGS

By

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ABSTRACT

Indoor air quality is described as the chemical, physical and biological characteristics of air in a residential or occupational indoor environment. In residential settings, there are many contributions to indoor pollution levels namely; human activities, biological sources and outdoor air. There has been increased focus on house dust due to its potential to contain biological and chemical pollutants in indoor environments. These have the potential to cause harm to human health. The purpose of this study was to conduct toxicological analysis of house dust collected from inside selected Durban residential buildings. The objectives of this study were to isolate, identify and quantify mould occurrence in house dust samples; to measure the occurrence of heavy metals (arsenic, lead and mercury) in house dust; and to analyse the cytotoxicity of house dust on human lung bronchus carcinoma epithelial line (A549) and human lung bronchus virus transformed epithelial cell line (BBM).

One hundred and five house dust samples were obtained from households that participated in the South Durban Health Study. In each home, a sample of settled dust was collected, using standardized protocols, then sieved and individually packed into polystyrene bags. The samples were taken from three surface areas namely; living room couches, bed mattresses, and carpets. Well documented methods were used for the isolation, identification and quantification of mould. The samples for heavy metals analysis were sent to Umgeni Water (chemistry laboratory, Pietermaritzburg) where standardised methods were used. Human cell lines were treated with five different dilutions of each house dust extract. Cell viability was assessed using the MTT assay. Toxic effects of house dust extract were analyzed, following house dust extract treatment and cells were stained with double dye (annexin-V- and propidium iodide) and analysed with flow cytometry, and fluorescent microscope. Cytokines were analysed by

Microbionix (Neuried, German) using a Luminex®100 plate reader for multiplex human cytokines analysis.

There were (n=128) mould types isolated and (n=105) were identified, of which (n=10) were predominately isolated moulds. This was further confirmed by Allerton Provincial Laboratory in Pietermaritzburg. Among the isolated genera in all three surface areas, *Rhizopus* spp and *Penicillium* spp were widely distributed throughout surface areas in greater proportion. The overall highest mean which was reported in this study and expressed in colony forming unit per gram (CFU/g) for *Penicillium* spp ranged (3400 – 62316 CFU/g) obtained from living room couches, followed by *Rhizopus* spp (5200 – 15990 CFU/g). The mould results were compared with the South African Occupational Health and Safety Act (OHSA) 85 of 1993 as amended suggested guidelines of 1,000, 000 CFU/g. The findings of this study suggest the moulds in the homes studied were below the suggested guideline. However, this does not imply that the indoor conditions are unsafe or hazardous. Instead, the findings act as an indicator of moulds presence indoors. The type of airborne mould, its concentration and extent of exposure and the health status of the occupants of a building will determine the health effects on an individual.

Heavy metals were detected in the dust in the following ascending order: arsenic (As) ranged from 1.3 μg/g -18.4 μg/g (mean, 4.26 μg/g), lead (Pb) ranged from 28.0-872 μg/g (mean 171.66 μg/g), and mercury (Hg) ranged from 0.6 -19.0 μg/g (mean, 2.22 μg/g). The mean concentration of lead in the dust was within the range of Canadian National Classification guidelines on residential contamination (500 μg/g). There was numerous numbers of samples in this study that exceeded these guidelines. The mean concentration of arsenic was within residential soil guidelines (20 μg/g). Mercury was within limits when compared with Global Hg project guidelines of soil/residential (6.6 μg/g), thought some of samples were notably above this mean. The ability of house dust extract to lower the cell viability which was slightly above 80% (prior treatment) to less than 50% (post treatment) in both cells was observed in this study. The

findings in this study showed that dust extract are toxic to human cell lines, and cells undergone a degree of apoptosis and necrosis 62% (A549) and 99% (BBM). The cytokines serve an important role in the non-specific defence external against insults. It was observed that A549 cells up-regulated the release of IL-6 and IL-8 pro-inflammatory cytokines and under-regulated the release of other cytokines analysed (IL-4, IL-13, and TNF-α). BBM cells released IL-4, IL-8 and IL-13 within limit of detection.

The presence of moulds in these sampled indoor household dusts, which is comparable with findings elsewhere indoors, show that moulds act as an indicator for building conditions such as dampness, which supports mould growth. Individuals, whether they are sensitized or not, may develop allergic reactions towards spores, thus the elevated numbers of spores quantified in this study are of concern. Some of the heavy metals reported in this study were higher or marginally higher than international norms and guidelines. The findings in this study strongly suggest that house dust extract is toxic to human lung cell lines. It must be noted, however, that this study may not reflect all that happens when a human lung is exposed to house dust. The findings of this study could contribute to the development of South African indoor air guidelines. In conclusion further study needed to be undertaken with respect to air pollution disease such as allergic; the reason being this study shown the reduced expression of cytokines that are involved in allergic inflammation.

DECLARATION

I **Bongani Alphouse Nkala** declare that: -

i. The research reported in this thesis, except where otherwise indicated, and is my original work.

ii. This thesis has not bee submitted for any degree or examination at any other university.

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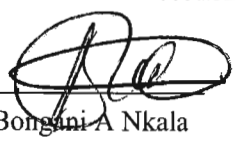

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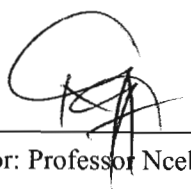

Supervisor: Professor Nceba Gqaleni	23/08/2010	**DURBAN**
	Date	Place

PRESENTATIONS

Nkala B and Gqaleni N. *Investigation of cytotoxicity of house dust on A549 and BBM cell line.* Presented at the Bio2Biz and South African Society for Microbiology Conference, 20-23 September 2009, ICC, Durban, South African.

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Nkala B and Gqaleni N. *Investigation of cytotoxicity of house dust on A549 and BBM cell line.* Presented at the 7th Congress of Toxicology in Developing Countries, 6-10 September 2009, Sun City, South Africa.

Nkala B and Gqaleni N. *The occurrence of heavy metals in house dust from selected Durban residential homes.* Presented at Joint Conference of the International Society for Environmental Epidemiology and the International Society for Environmental Epidemiology and the Exposure Analysis, 12-16 October 2008, Pasadena, California, USA.

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Nkala B and Gqaleni N. *The occurrence of heavy metals in house dust from selected Durban residential homes.* Presented at 4th Public Health Association of South Africa Conference, 2-4 June 2008, Cape Town, South Africa.

Nkala B and Gqaleni N. *The isolation and identification of indoor air pollutants from selected Durban residential buildings*. Presented at the South African Veterinary and Paraveterinary Congress, 5-7 June 2006, ICC, Durban, South Africa.

LIST OF ABBREVIATIONS

A549	Human lung bronchus carcinoma epithelial cell line
AD	Actinomycin D
As	Arsenic
ASHRAE	American Society of Heating, Refrigerating and Air-Conditioning
ATSDR	Agency for Toxic Substances and Disease Registry
AQC	Analytical quality control
BBM	Human lung bronchus; virus transformed epithelial cell line
BEGM	Bronchial epithelial growth medium
BRI	Building related illness
CFU	Colony forming unit
CHX	Cycloheximide
CO_2	Carbon Dioxide
COPD	Chronic obstructive pulmonary disease
CV	Coefficient Variance
DG18	Dichloran-glycerol agar
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPX	p-Xylene –bis-Pyridinium bromide
EHRS	Environmental Health and Radiation Safety
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMEM	Eagles- Minimum essential medium
EPA	Environmental Protection Agency
ETS	Environmental Tobacco Smoke
FBS	Faecal Bovine Serum

HBBS	Hank's Balanced Salt Solution
HDM	House Dust Mites
HEPES-BSS	HEPES-Buffered Saline Solution
HMRC	Heavy Metal Remediation Committee
Hg	Mercury
IAQ	Indoor Air Quality
IARC	International Agency for Research on Cancer
IL	Interleukin
IQ	Intelligence quotient
IOM	Institute of Medicine
IUIS	International Union of Immunological Societies
LDH	Lactate dehydrogenase
LLOQ	Lower limit of quantification
LPB	Lacto-Phenol Blue
LPS	Lipopolysaccarides
MEA	Malt Extract Agar
MFI	Mean Fluorescence Intensity
MHRA	Manufactured Housing Research Alliance
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole
NEAA	Non-essential amino acid
NHLBI	Heart, Lung and Blood Institute
NIOSH	National Institute for Occupational Safety and Health
OAA	Ontario Association of Architects
OHSA	Occupational Health and Safety Act 85 of 1993 as amended
Pb	Lead
PM	Particulate matter

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PI	Propidium iodide
PS	Phosphatidylserine
ROFA	Residual old fly ash
RNA	Ribonucleic acid
SBS	Sick building syndrome
SD	Standard deviation
SDHS	South Durban health study
TNF	Tumor necrosis factor
UNIDO	United Nations Industrial Development Organization
VOC	Volatile Organic Compound
XTT	Tetrazolium salt
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW

1.1 Introduction

This study compliments the dissertation of Jafta (2007), which described in detail, the indoor air quality of selected schools and residences in Durban. Jafta (2007) was responsible for the sampling of house dust, in the selected Durban residential homes, used in this study. Thus, the present study provides an in-depth analysis of the nature of pollutants in house dust. Previous work conducted in South Durban households reported high asthma prevalence in children in the South Durban Health Study (SDHS) (Jeena *et al.,* 2002; groundwork, 2003). It is believed that asthma is associated with indoor pollutants, and this gives us a good motivation to study house dust pollutants as potential contributors to poor indoor air quality.

House dust is a complex mixture of various bio-contaminants and a major source of allergens in non-industrial indoor environments (Jacob *et al.*, 2002). Therefore, it is often difficult and expensive to obtain a precise measurement of each relevant component to estimate the risk causing respiratory problems (Jacob *et al.*, 2002). House dusts that are taken from different locations at different times may vary profoundly in composition, which further complicates dust for pollution investigation. House dust constituents such as micro-organisms and heavy metals, may be toxic to humans and presently, no specific strategy and protocols in place for determining its toxic effects. The toxicity of dust has been studied by various researchers in the laboratory using various cytotoxic methods (such as LDH, XTT, MTT, Annexin-V etc) in order to quantify its effects (Roepstorff and Sigsgaard, 1997, Mathiesen, 2003; Mahedevan *et al.*, 2005).

1.2 Aim and objectives

The aim of this study was to conduct a toxicological analysis of house dust collected from inside selected Durban residential buildings.

The objectives of the study were:

i) To isolate, identity, and quantify mould occurrence in house dust samples.

ii) To investigate the occurrence of heavy metals namely: mercury (Hg), arsenic (As) and lead (Pb) that are associated with indoor air pollution.

iii) To investigate the cytotoxicity of house dust extract on human lung bronchus carcinoma epithelial cell lines (A549) and human lung bronchus virus transformed epithelial cell line (BBM).

1.3 Review of related literature

Indoor air quality (IAQ) is described as physical, chemical and biological characteristics of air in an indoor (residential or occupational) environment. In residential settings there are many contributors to indoor pollution levels, namely human activities (man-made), biological sources and outdoor air. Mitchell *et al.*, (2007) studied factors that are associated with human health such as building designs in terms of adequate ventilation, chemical and biological factors. Shendell *et al.*, (2004) categorized indoor sources as being physical, chemical and biological contaminates with toxic and allergic properties.

The physical characteristics of air within an indoor environment include relative humidity, temperature and light, is very important for the well being of occupants (ASHRAE 1999; WHO 1999). Recently, IAQ has become increasingly important in industrialized countries due to the fact that people spend more than 90% of their time indoors (Kmucha, 2000), which makes them susceptible to illness related to airborne contaminants (Pahwa, 1995).

The chemical exposures are anthropogenic (man-made) products that people are exposed to indoors include environmental tobacco smoke (ETS), gaseous products, such as nitrogen oxides, sulphur oxides, ozone and other gases like volatile organic compounds (VOC), and airborne particles in a form of particulate matter ($PM_{2.5}$ and PM_{10}).

Biological agents, such as allergens from house dust mite, cockroach and mould in air and dust; and airborne fungal propagules are also found in high levels indoors. Exposure to these agents is linked to adverse health (IOM 1993; IOM 2000). The concentration of some of these air pollutants indoors may be up to hundred times higher than concentration outdoors because of the sources and the characteristics of indoor environment (WHO 1999). Many biological and non-biological agents contaminate the air in homes, offices, or other indoor environment (Berglund *et al.*, 2004; Srinkanth *et al.*, 2008). Human health requires a good indoor air quality thus clean air is essential for good health (Fanger, 2001). Human health has been compromised by the indoor pollutants which have acted as a major contribution towards poor health conditions (Brunekreef and Holagate, 2002). Progress has been made over the years in terms of focusing on the investigation of indoor pollutants and their health effects on the indoor occupants: namely, dust mite allergens, cockroaches, moulds, particular matter (PM), heavy metals, etc (Sneller and Roby, 1979, Jacob *et al.*, 2002, Bisht *et al.*, 2003, O'Connor *et al.*, 2004, Dasgupta *et al.*, 2006; Sarnat and Holguin, 2007). Epidemiologists have suggested that health effects are directly proportional to the exposure patterns (Lee, 1997, Gauderman *et al.*, 2000, Smith, 2000, Ezzati and Kammen, 2001, Rahman *et al.*, 2001, Donohoe, 2003; Zhang and Smith, 2003). Health effects alone cannot be used to determine the extent of pollution levels, because the time occupants spend in the polluted environment needs to be taken into consideration as well (Bruce *et al.*, 2000). Pollutants vary in their potency and ability to cause detrimental health effects. For example some pollutants may cause temporary eye or lung irritation whereas others are

implicated in playing a role in chronic conditions such as cardiovascular disease (Kumar *et al.*, 2005).

1. 3.1 Physical contaminants

In the understanding of IAQ, it is imperative to discuss building related factors, such as heating, ventilation, air conditioning and household activities (Loftness *et al.,* 2007). The physical environment which is controlled by an indoor climate, namely humidity, temperature and light, is very important for the well being of occupants not only for comfort but for influencing pollutant levels (Health Canada, 1995; Avgelis and Papadopoulos, 2004). The reason being dampness and mould are directly associated with building construction and human health. In other words the indoor climate is very important in maintaining indoor air quality (Loftness *et al.,* 2007).

The issues concerning heating which interfere with the indoor air quality equilibrium such as the use of bio-fuels like wood, coal and paraffin, have been addressed somewhere else to minimize indoor air pollution. This has been done by the introduction of insulation in both formal and informal houses which can be attributed to the depletion of natural resources as a result of high space heating requirements. Lombard *et al.*, (1998) have pointed out that such indoor pollutants are undesirable for occupants and poses serious health risk. Matthews and van Wyk, (1996) have recommended the use of clay bricks for the construction of new low cost houses and integrate it with ceiling for insulation purpose as to save on energy and address the indoor air quality issues in this nature.

Jia *et al.*, (2008) identified key products that are source of indoor air pollution, namely, furniture, flooring, paints, coating, adhesives, sealants, wall coverings, wood products, textile, insulation, and cleaning products. The environmental building, which is free from moulds, is what is required to maintain a good balance for IAQ. Roulet, (2001) suggested the importance of

controlling indoor mould sources which is the challenge, because one needs to control moisture in existing buildings (Figure 1), and the domestic source of moisture (Figure 2) which include bathing, showering, cooking, mopping, and clothes washing and drying. The most problematic source is unvested gas appliances, indoor gardens, saunas, hot tubs, and indoor storage of firewood (MHRA, 2000). Shadwell *et al.*, (2000) state that air-conditioners are the major source of dampness in indoor environment and are a good support medium for mould growth and that an increase in wall dampness exacerbates this problem. Dampness and mould are complex problems from the point of view of buildings construction, and human health, respectively. It is necessary to control dampness and moisture, which exacerbate to fungal growth that results into mould infestation (Loftness *et al.*, 2007). The presence of visible mould indoors has a negative impact on occupants because they become susceptible to illnesses that are building related illness (Nevalainen and Seuri, 2005).

Building related illness (BRI) is the term used when symptoms of diagnosable illness are identified and can be attributed directly to airborne building contaminates; occupants complain of symptoms such as cough, chest tightness, fever, chills and muscle aches (Fanger, 2001). The symptoms can be clinically defined with identified causes but reduction in the symptoms may require prolonged recovery times after leaving the building (Pahwa, 1995). In sick building syndrome (SBS), also called building related symptoms; occupants complain of acute discomfort, such as headache, eye, nose, or throat irritation, dry cough, dry or itchy skin, dizziness and nausea, difficulty in concentration, fatigue, and sensitivity to odours (Bachmann and Myers, 1995; Engvall *et al.*, 2001). Generally the symptoms disappear after the affected person leaves the building (Gupta *et al.*, 2007).

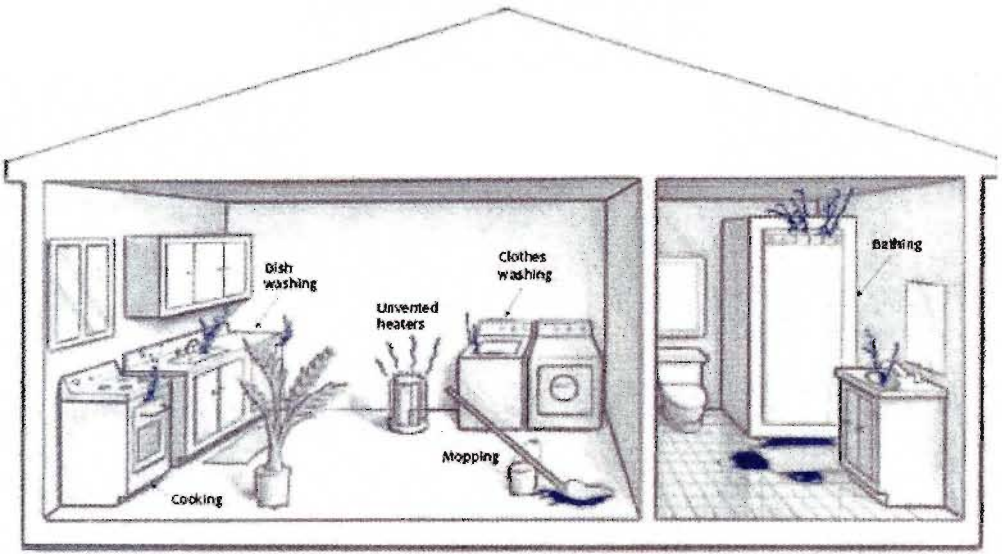

Figure 1: Moisture production from indoor activities (MHRA, 2000)

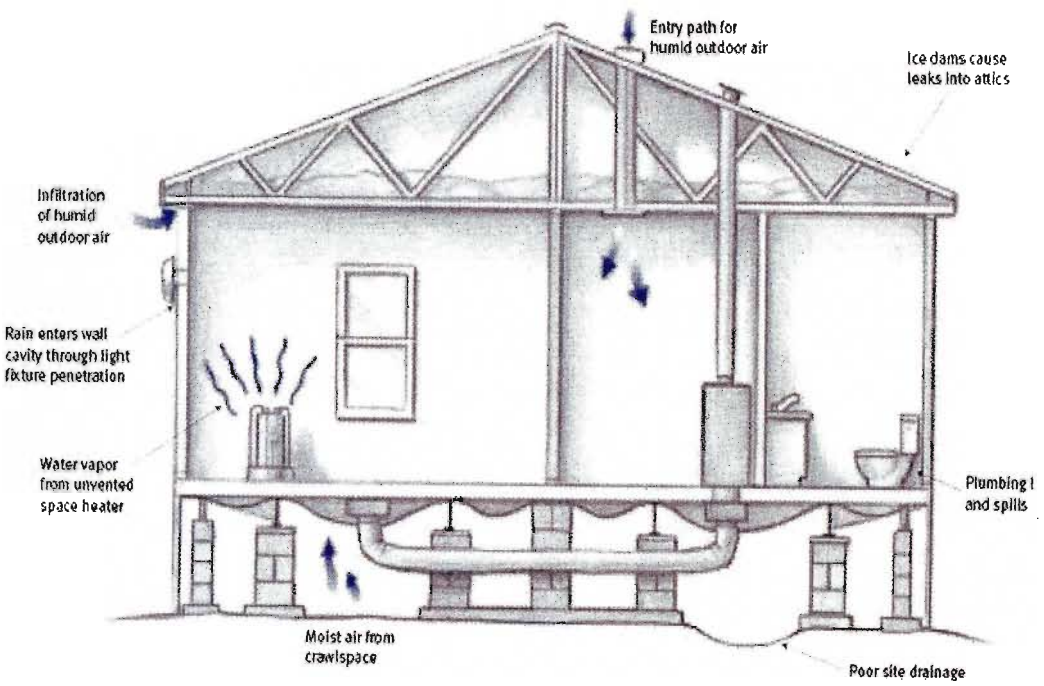

Figure 2: Sources of indoor moisture (MHRA, 2000)

1.3.2 Chemical contamination

Chemical sources of contamination include formaldehyde, radon, and volatile organic compounds which emanate from soil, solvents, paint, varnishes, carpet, and pesticides. These contaminants cause long and short term illness (Pahwa, 1995). Heavy metals have been reported to be one category of the indoor pollutants that interfere with the well being of occupants (Cedric and Heike, 2005).

1.3.2.1 Radon

Radon is a product of radioactive decay process beginning with uranium – 238 and thorium -232. This gas is invisible, odourless and tasteless (EHRS, 2008). It is naturally found on earth during the breakdown of uranium in soil, rocks, and water and gets into the air we breathe (USA EPA, 1995). Radon gas can enter buildings such as offices, schools, and homes through cracks or other openings in the foundation (Saravanan, 2004). USA EPA, (1995) estimated that radon causes about 14,000 deaths per year in the United States of America (USA). However, this number could range between 7,000 to 30,000 deaths per year. Furthermore lung cancer is predominately associated with radon exposure and is the second largest cause of lung cancer among non-smokers in the USA (EHRS, 2008).

1.3.2.2 Formaldehyde

Formaldehyde is a colourless, flammable gas at room temperature, and is also known as methylene oxide, oxymethylene, methylaldehyde, and oxomethane (ATSDR, 1999). Formaldehyde has number of industrial uses, such as in the production of fertilizer, paper, and construction materials such as particles boards, plywood and urea-formaldehyde foam insulation (ATSDR, 1999). In other instances, indoor occupants have been reported to be indirectly affected by formaldehyde which is emitted by biocides (which are used in paints as

preservatives) that are used as paints additives (Salthammer, 2004). It can also be naturally produced by our bodies as part of our normal, everyday metabolisms and does not pose any harmful effects in this fashion (Saravanan, 2004). Formaldehyde is irritating to tissues when it comes into direct contact with them, and it can also be harmful to some people while harmless to others (USA EPA, 1995; Offit and Jew, 2003).

The most acute symptoms include irritation of the eyes, nose, and throat, along with increased tearing, which occurs at elevated concentrations in the air greater than 20 ppm and these can trigger asthmatic attacks in people (USA EPA, 1995, Saravanan, 2004; ATSDR,1999). Furthermore, association of formaldehyde and asthma have been reported which focused on wheeze and nocturnal attacks of breathless (Zhao *et al.*, 2008).

1.3.2.3 Volatile Organic Compounds (VOCs)

Volatile organic compounds are ubiquitous indoors but some are present in outdoors environment together with specific outdoor inorganic compounds, for example ozone (Wolkoff and Nielsen, 2001). VOCs are released from burning fuel, such as petrol, wood, coal, or natural gas (EHRS, 2008). Batterman *et al* (2007) investigated the correlation of VOCs emitted from garages, which has been reported to be higher than what has been reported in houses that are attached to garages. Garages have been identified to be a source of VOCs contamination into the houses. Many VOCs are emitted by construction materials, furnishings and consumer products such as latex paints, cleaning agents, household solvents, detergents, waxes and varnishes (Wolkoff and Nielsen, 2001, State of Knowledge Report, 2001; Saravanan, 2004). Jia *et al.*, (2008) reported high levels of benzene indoors compared to outdoor levels and which was categorised by International Agency for Research on Cancer (IARC) as type 1 carcinogen which was noticeable in garages (outdoor source), and also in new and renovated buildings (indoor source).

Human exposure to VOCs can result in a number of discomforts ranging from acute effects (tissue reaction in the eyes, nose, airways and skin), sub-acute effects (headache, drowsiness, pain and inflammatory responses) and chronic effects caused by absorption or metabolism of VOCs (Wolkoff and Nielsen, 2001). In other instances people can be exposed to high concentration of VOCs which can cause liver damage, kidney, cancer, asthma and central nervous system irregularities (Delfino, 2002; Safe Air, 2008).

The international standards and/or guidelines of common indoor pollutants that are associated with the indoor pollution have been documented by Charles *et al.*, (2005) (see Table 1).

Table 1: International standards and/or guidelines for common indoor contaminants (Charles et al., 2005).

	EPA	Canada	WHO/EU	Hong Kong	Germany
Carbon Dioxide		3,500 ppm [L]		88/1000 ppm [8 hr]	
Carbon Monoxide	9 ppm (Max)	11 ppm [8 hr]	90 ppm [15min]	1.7/8.7 ppm [8 hr]	52/5.2 ppm [0.5 h]
	35 ppm [1hr]	25 ppm [1 hr]	50 ppm [30 min]		13/1.3 ppm [8 hr]
			25 ppm [1 hr]		
			10 ppm [8 hr]		
Formaldehyde		0.1 ppm [1 hr]	0.081 ppm	0.024/0.081 ppm [8 hr]	
		0.05 ppm [L]	0.1 mg/m^3 [30 min]		
Lead	1.5 $\mu g/m^3$ [3 months]		0.5 $\mu g/m^3$ [1 yr]		
Nitrogen Dioxide	0.05 ppm[1 yr]	0.05 ppm	0.1 ppm [1 hr]	0.021/0.08 ppm [8 hr]	0.19 ppm [0.5 h]
		0.25 ppm [1 hr]	0.004 ppm [1 yr]		0.03 ppm [1 wk]
Ozone	0.12 ppm[1 hr]	0.12 ppm [1 hr]	0.064 ppm	0.025/0.061 ppm [8 hr]	
	0.08 ppm		(120 $\mu g/m^3$) [8 hr]		
Particles <2.5µm	15 $\mu g/m^3$ [1 yr]	0.1 mg/m^3 [1 hr]			
	65 $\mu g/m^3$ 24 hr]	0.04 mg/m^3 [L}			
Particles <10µm	50 $\mu g/m^3$ [1 yr]			0.02 / 0.018 mg/m^3 [8 hr]	
	150 $\mu g/m^3$ [1 yr]				
Sulphur dioxide	0.03 ppm [1 yr]	0.38 [5 min]	0.048 ppm [24 hr]		
	0.14 ppm [24 hr]	0.019 ppm	0.012 ppm [1 yr]		
Radon	4 pCi/L [1 yr]		2.7 pCi/L [1 yr]	4.1/ 5.4 pCi/L [8 hr]	

1.3.2.4 Heavy metals

Heavy metals can be found in old or restored houses, firearms, some workshops, and latex paints (Volesky and Holan, 1995). Other sources of heavy metals, that have been studied globally which contribute to indoor air pollution and human exposure, are street dust, industrial dispersion, type building deterioration, and automobile traffic near homes and type of heating (eg. coal) used in homes during heating and cooking (Thornton *et al.*, 1990, Meyer *et al.*, 1999, Harper *et al.*, 2003, Davis and Gulson, 2005, Ferreira-Baptista and Miguel, 2005, Lee *et al.*, 2005; Shinggu *et al.*, 2007). The presence of heavy metals in dust particles can cause a number of chronic toxins even at relatively minor levels and their tendency to accumulate in selected tissues of human body have been reported (Howard, 2002). Minor levels can be in a form of dust; and dust metals may travel from roads, through the windows and balconies into the houses (Soltan *et al.*, 2005).

Minute particles of heavy metals suspended in the air, which are often called a component of particulate matter (PM), are present as a result of both natural and human activities (Brigden *et al.*, 2002). Particulate pollution is implicated in the worsening of respiratory illness such as asthma, and increasing premature mortality from respiratory and heart disease (Sarnat and Holgiun, 2007). The main cause of the small particles being implicated with respiratory illness is that they are small (0.5-5 μm) and are inhaled into lungs (Figure 3) and retained deep in the lungs (Meyer, 1983, Rahman *et al.*, 2001; Bridgen *et al.*, 2002). In particular, particulates sized <0.1 μm termed ultra-fine particles, are of greatest concern with regards to adverse human health; reason being they are trapped in the larynx and bronchial tubes before they reach the lungs (Howard, 2002; Bridgen *et al.*, 2002). Larger particles (10 μm) that are preventable from reaching the deep airways by the respiratory system's protective mechanism are more likely to enter the lung during inhalation (Small, 2002). When airborne particles from either outdoor or indoor origin are inhaled they are deposited when they come in direct contact with various

surfaces of the respiratory track (Allermann *et al.*, 2002; Nevalainen and Seuri, 2005). Fly ash particles are generally composed of stable elements or compounds that are usually not considered directly toxic in concentration found in ambient air but subtle toxicity has been recognised under different condition (Casarett and Doull, 1975).

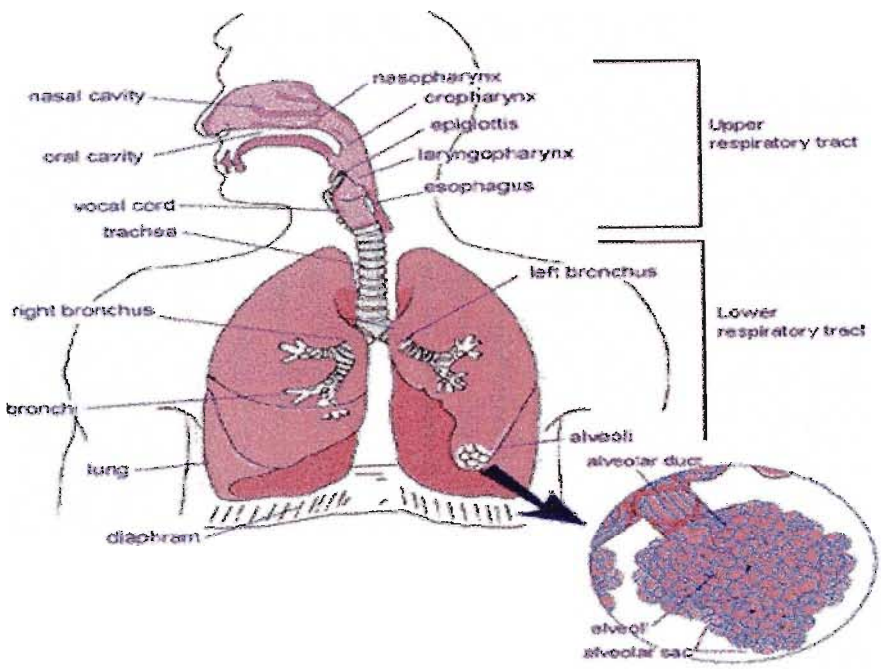

Figure 3: The human respiratory system (Porter, 2006).

Heavy metals, such as mercury (Hg), lead (Pb) and arsenic (As), are well-known for their ability to interfere with human health (Weaver *et al.*, 1998, Järup, 2003; Ljung, 2006). Researchers have studied heavy metals' ability to cause chronic effects on humans and their potential environmental health hazard, especially in infants and young adults since their respective immune systems are not yet fully developed (Hough *et al.*, 2004, Ibrahim *et al.*, 2006; Neuberger *et al.*, 2008). Heavy metals exposure can occur through a variety of routes: they can be inhalable vapour form, or they may be ingested involuntarily through food and drink. When metals are absorbed they can be distributed in tissues and organs (Howard, 2002, Liu *et al.*, 2002; Caceres *et al.*, 2005). In the present study problematic heavy metals, such as Hg, Pb and As that have been associated with the air pollution are further discussed.

1.3.2.4.1 Mercury (Hg)

Mercury (Hg) is an extremely toxic, non-essential trace element having no biochemical or nutritional function (Goodman, 1974; Huisingh, 1974). Biological mechanisms for its removal are poor. It can exist in three main forms: metallic (as a liquid or vapour), ionic salts and, in organic compounds (Okoronkwo *et al.*, 2006). The Hg salts show a high acute toxicity, with a variety of symptoms and damages (Chapman and Chan, 2000). Metallic Hg has many applications in the electrical industry (e.g. alkaline batteries, electrical switches, fluorescent lamps), in medical equipments (e.g. thermometers, electroanalysis, gauges, manometers, barometers) and in dental amalgams (Roberts *et al.*, 2001, Reto, 2002; Mukherjee *et al.*, 2004). In the chemical and mining industries, Hg is used as catalysis in reactions to form polymers, in manufacturing chlorine and caustic soda, and in extracting gold from ore (Dujardin *et al.*, 1998). Mishandled or spilled Hg, from devices used in the home or workplace, is often the source of unintentional exposure (Alloway, 1990).

The primary Hg route of exposure includes oral ingestion, dermatologic absorption, and inhalation (Moody and Chu, 1995; Henderson *et al.*, 2001). The main toxicity of Hg exposure occurs through inhalation of the vapour form which is absorbed by pulmonary circulation and distributes the Hg to the brain, kidneys, gut and lungs (Zalups and Lasha, 1994, Ratcliffe *et al.*, 1996; Goyer, 1996). Globally, Hg has increased importance as being a widespread contaminant (Fitzgerald *et al.*, 1998; Carpenter *et al* 2000). Mercury dispersion through atmospheric deposition has increased markedly through waste incineration. Ironically the medically industry is one of the largest contributors towards Hg contamination in this fashion (Mukherjee *et al.*, 2004, Palmer *et al.*, 2006; Hodgson *et al.*, 2007). At this stage there are no standards and regulation concerning safeguarding residential occupants from Hg exposures from settled house dust. However, Global Mercury Project (2003) has come up with some guidelines as to safe guide the levels of Hg in soil/residential which is 6.6 μg/g Hg (UNIDO, 2003).

1. 3.2.4.2 Lead (Pb)

Lead (Pb) can be termed as ubiquitous, which can be found in the air, dust, and environment, and is essentially of an anthropogenic origin when viewed as a contaminant (Komarnicki, 2005; Filippelli *et al.*, 2005). Lead released into the environment has a long residence time compared with other pollutants (Alloway, 1990). It can contaminate soil, air, drinking water, and foods, thereby posing a threat especially to infants, whose digestive systems absorb lead at significantly higher rates than adults (Creel, 2002). In order for Pb to causes harm it must primarily enter the body through ingestion, inhalation or dermal absorption (Mahaffey, 1997). Levels of Pb found in air, food, water and soil/dust vary widely throughout the world and depend upon the degree of industrial development, urbanisation and lifestyle factors (Schwela, 2001).

Lead toxicity has been studied globally due to its industrial dispersion and widely spread in the environment. It has been used as additives in ceramics, water pipes, solders, pigments and paints and it is widely spread in the environment (Romieu *et al.*, 1995; Harper *et al.*, 2003). Studies conducted in South Africa and Africa at large; before Pb was banned (2006) as an additive, which was a major source of Pb contamination and poisoning, reported that there were elevated blood lead levels in children who resided near industrial areas and within the vicinity of heavy traffic sites (Nriagu *et al.*, 1996, Harper *et al.*, 2003; Shinggu *et al.*, 2007). Lanpher *et al.*, (1989) reported the association of blood lead levels in children, which was directly proportional to the floor dust elevation suggesting that children were at high risk from Pb exposure. Liggans and Nriagu (1998) and Mathee *et al.*, (2002) found correlations between blood lead increase and use of lead-based paint indoors. Researchers are now more concerned with the use of Pb as an additive in paints and this has resulted from postulations that lead-based paint is a major contributor towards human Pb exposure. Furthermore, its detrimental health effects have been implicated in children (Röllin *et al.*, 2007; Mathee *et al.*, 2007).

Indoor Pb poisoning has received global interest for a number of decades. Interest in petrol as a source of pollutants has shifted due to the ban of lead based petrol usage in vehicles certainly by most countries. Lead-based paint and other local industries, such as smelting batteries, have been pin-pointed as the major contributors towards indoor pollution thus pose threats to human health and more specifically children's health as this can be seen by their blood levels elevations (Albalak *et al.*, 2002, Meyer *et al.*, 2003; Brown *et al.*, 2005). Epidemiological studies that have been conducted globally with regards to Pb effects on children's health, such as neurological and behavioural problems in children, extend to raise hearing thresholds and a decrease in intelligence quotient (IQ). Low blood lead concentration has been linked to acute encephalopathy, memory loss, and death at high blood concentrations (Manser *et al.*, 1989, Shen *et al.*, 2001; Creel, 2002). In the residential settings, Britain Greater Council has suggested guidelines of 500 μg/g Pb, and this is purely for justification and investigation purposes. When Pb is >500 μg/g, it increases the need for control (Thornton *et al.*, 1990).

1.3.2.4.3 Arsenic (As)

Arsenic (As) is a ubiquitous element in the environment and widely distributed in the earth's crust (Buat-Menard *et al.*, 1987, Trepka *et al.*, 1995; Ng *et al.*, 2003). The major sources of As in the environment are metal ores and coal-fired power plants. It is released during smelting process and/or in coal-burning which produces stacks dust and flue gas to contaminate the soil and water with As and pesticides (Roy and Saha, 2002; Wang and Mulligan, 2006). Arsenic has an industrial use in common products such as wood preservatives, pesticides, herbicides, fungicides, and paints (Howard, 2002; Ratnaike, 2003). In the environment it combines with oxygen, chlorine, and sulfur to form inorganic As compounds (ATSDR, 2007). Inorganic As has been identified as a global concern with reference to significant hazards in a population, especially in the developing countries (Liu *et al.*, 2002; Li *et al.*, 2006).

Human exposure to As can be via air, food and water (Liu *et al.*, 2002; Caceres *et al.*, 2005). Arsenic has been classified as an human carcinogen and health concern due to the fact that it causes chronic poisoning in humans; it has a short half-life in the body (weeks) but its effects can be seen years after exposure has ceased (HMRC, 2003). Chronic As exposure also causes a markedly elevated risk for developing various cancers; the most notable ones are skin cancer, cancer of liver, lung, bladder, and possibly the kidney and colon (Pershagen, 1981, Mushak and Crocetti, 1995; Ng *et al.*, 2003). The primary target organs of As poising are the gastrointestinal tract, brain, and kidney; eventually the skin, bone marrow and peripheral nervous system are also affected (Tchounwou *et al.*, 2004).

The other health effects caused by As are stomach ache, nausea, vomiting and diarrhoea (Ljung, 2006). Acute As poisoning is infamous for its lethality which stems from arsenic's destruction of the integrity of blood vessels and gastrointestinal tissue and its effect on the heart and brain (Benramdane *et al.*, 1999). Currently there are no As standards in place with respect to the settled dust as to regulate the dispersion of it in the indoor environment in order to minimise effects on human health. However, the South African Occupational Health and Safety Act (OHSA) lists suspended guidelines of <10 $\mu g/m^3$ for permissible exposure (Peters *et al.*, 1986; NIOSH, 1995). The above-mentioned guidelines cannot be directly utilised as to benchmark As in settled dust due to the fact that it is mean't for suspended dust particles. The available guidelines that can be used as a benchmark of As determined in this study in the residential soil is 20 µg/g (Tsuji *et al.*, 2005).

1.3.3 Biological contamination

Indoor house dust may contain biological contamination such as pollen, moulds, bacteria, viruses, cockroaches, dust mites and epithelial cells (Smith *et al.*, 2000; Derek *et al.*, 2004). Contaminants may enter a building in various ways: through open doors, windows, ventilation

shafts or by becoming attached to pets, cloths, or other personal items that are taken into a building (Curits *et al.*, 2004). Previously, it was thought and understood that inhaled allergens were derived from pollen but the latest developments point to dust extracts (Platts-Mills *et al.*, 2000). Gram-negative bacteria have endotoxin in the outer membrane of the cell wall which is a heat stable lipopolysaccharide (LPS) and is released when the bacterium lyses, or during its growth, and is toxic to the host (Prescott *et al.*, 1996). It has been shown that LPS affects macrophages and monocytes by binding to specific plasma proteins called LPS-binding proteins. The LPS-binding protein complex then attaches to receptors on monocytes, macrophages, and other cells and this triggers several events, including the production of cytokines IL-1, IL-6, and tumour necrosis factor (TNF) (Prescott *et al.*, 1996). Dust mites and cockroaches have been studied worldwide, and have been recognized as predominant allergens in the indoor environment, especially in urban dwellings (Kalpakl and Turkey, 2001, Macan *et al.*, 2003; Wood, 2004) and their ability to release cytokines have been studied. In this literature review the biological contaminates that is involved in the allergens indoor dwellers, namely dust mites, cockroaches, mould and epithelial cells are discussed.

1.3.3.1 Dust mites and cockroaches

Dust mites and cockroach allergens have been noted to fall rapidly after domestic disturbance such as vacuum during cleaning, bed making, etc, (Platt-Mills, 2007). Becher *et al.*, (1996) discussed indoor factors that exacerbate respiratory hypersensitivity and asthma indoors dwellers. These factors consist of allergens from house dust mites, pet dander, insects such as cockroach, and moulds. Cockroach allergens exposure to children in the inner city has been reported as being high in clinical asthma cases (Belanger *et al.*, 2002; Gruchalla, 2005). Furthermore the incidence of asthma in the inner city in the USA tends to be more frequent and severe in children (Busse and Mitchell, 2007).

Cockroaches have been termed the major indoor allergen that can pose a serious risk of respiratory diseases such as allergic asthma in sensitized individuals (Lehrer and Reese, 1998). Gold *et al.*, (1999) could not find any association between cockroaches and wheeze in children, and moreover, no association with bronchial inflammation. Litonjua *et al.*, (2001) studied the relationship between indoor allergens and the development of doctor-diagnosed asthma which they investigated, and the relationship between exposure to these allergens and recurrent wheezing. In their results the relationship between asthma and recurrent wheezing and low levels of cockroach allergen were observed.

There are conflicting reports in literature on health. Breysse *et al.*, (2004) have reported on the residential risk factors of indoor allergens exposure which have been associated with asthma exacerbation in children. Lau *et al.*, (2000) have linked asthma with immediate hypersensitivity to dust mites and cockroach allergens as being the major risk factor for doctor-diagnosed asthma. Immediate hypersensitivity to indoor allergens is considered to be causally associated with asthma exacerbations. Tunnicliffe *et al.*, (1999) examined sensitivity and exposure status of subjects, with severe and mild asthma, to mite, cat and dog allergens. The results showed allergens were higher in the sensitized severe asthmatics compared to the sensitized mild asthmatics. Other researchers reported lower exposure levels of the other allergens, such as dust mites, and dogs, and their prevalence in the indoor environment which entirely depends on the housing and locations (Rosenstreich *et al.*, 1997, Leaderer *et al.*, 2002, Belanger *et al.*, 2002; Gruchalla, 2005). It can be noted that no association was found between cats allergen concentrations and risk but repeated wheeze has been reported (Gold *et al.*, 1999). In other cases, it was found that a number of children had high exposure to cat allergens with asthmatic risk, but no allergy was reported (Platts-Mills *et al.*, 2000; Platts-Mills *et al.*, 2001).

Results suggest that high indoor allergen exposure is genetically controlled since exposure appears to be protective for some children and a risk factor for others. Lau *et al.*, (2000) confirmed that not only indoor allergen exposure exacerbates asthma but that genetic and other environmental factors are important for the development of asthma phenotypes which plays an important role in influencing the structural abnormalities regarding growth and elasticity of airways and lung parenchyma.

Cabana *et al.*, (2004) studied environmental factors that trigger asthma in patients. The factors included *inter alia* plants (such as trees, weeds, hay), animals danger (dogs, cats, others) dust (dust mites, stuffed animals, others), etc but were of no benefit to the current National Heart, Lung and Blood Institute asthma guidelines. Asthma has received a deal of great attention lately because it has been classified into allergic diseases such as hay fever which affect the respiratory tract (Platts-Mills *et al.*, 1998). Researchers have studied environmental factors and established the house dust mite allergens that are responsible for exacerbation of asthma (Lau *et al.*, 2000).

1.3.3.2 Moulds

Moulds are naturally occurring filamentous fungi. Most moulds are saprotropic which means they need substrate such as (wood, paper, paints, soft furnishing, potting soil, dust, slum scales and food) to reproduce (Bayer *et al.*, 1999, Albright, 2001; Summers *et al.*, 2003). Some moulds require a constant source of standing water, while others can thrive on the relative humidity (Prescott *et al.*, 1996). The relative humidity of an air-water mixture is defined as the ratio of the partial pressure of water vapor in the mixture to the saturated vapor pressure of water at a prescribed temperature. Relative humidity is normally expressed as a percentage and is defined in the following (Perry and Green, 1990). Once they have a nutrient base and water, mould species produce spores that can survive harsh environmental conditions, such as dry conditions that do not normally support mould growth (OAA, 2003).

They reproduce either sexually or asexually and spread through the distribution of spores. The rare reproduction is sexual reproduction which requires compatible strains of opposite sex for mating. Asexual reproduction occurs through production of spores and develops in sporangia at the tip of the aerial hyphae spread (Prescott *et al.*, 1996). Developed spores are dispersed by wind. The indoor environment plays a crucial role in support of mould growth. A warm indoor environment with relatively humidity greater than 50% is optimum for indoor moulds growth (Deacon, 1997). Usual sources of moisture include kitchens and bathrooms which tend to have increased mould growth (Verheoff and Burge, 1997). The most commonly identified indoor moulds species include *Cladosporium*, *Penicillium*, *Alternaria*, *Aspergillus* and *Mucor* (Ren *et al.*, 2002; Beguin and Nolard, 2006). Zureik *et al.*, (2002) reported that adults living in different countries who were sensitized to *Alternaria* and *Cladosporium* have shown to developed severity asthma.

Mould pollutants effects are normally manifested in specific target organs (European Communities, 1991). These effects may be direct as the pollutions come into intimate contact with the organ affected. Such is the case for mild eye irritation to mortality and making a person more susceptible to infection or to the development of a chronic respiratory disease (Stern *et al.*, 1984). Effects may also be indirect. For example, pollutants may enter the bloodstream from the lungs or distant to the intimate contact with air contaminants. Air pollution principally affects the respiratory, circulatory, and olfactory systems. The respiratory system is the principal route of entry for air pollutants, some of which may alter the function of the lungs (Godish, 1985; Bency *et al.*, 2003).

1.3.3.3 Epithelial cells

Epidemiological studies have reported health problems that are associated with inhalation of house dust. Its impact may range from slight irritation of the eye and mucus membranes of the throat but can also advance allergy, asthma and bronchitis (Mills *et al.*, 1999; Holgate *et al.*, 2000). Laberge and Bassam (2004) have reported on the role of the epithelial cell as effectors in asthma due to being in a key position to interact with the external environment. The exposure characteristics are not sufficiently understood to allow a reliable dose-response assessment of indoor exposure to toxigenic microbes or any single toxin. The possible presence of one of the dust pollutants may trigger inflammatory response with multifactor causes with difficulties in isolating single parameter being responsible (Allermann *et al.*, 2002). In this regard the availability of information sufficient enough to suggest the important chain of events leading to potentially harmful exposure. Furthermore, the use of *in vitro* methods for screening of the biological hazards of organic dust, regardless of its constituents and concentration, will make it easy to rank individual samples according to their given parameters such as inflammatory potential, etc (Allermann and Poulsen, 2000).

Recently, epithelial cells have received attention in mechanistic studies of air pollution-induced airway disease. The findings of these studies suggest that cells are likely to play a fundamental role in the pathogenesis. The reason for this is that bronchial epithelial cells play an important role as a physical barrier in protecting the underlying tissue and maintaining the local environment in the airways (Mills *et al.*, 1999). Epithelial cells differ within the airways due to their functionality, as the columnar ciliated and goblet cells are the most prominent. Ciliated cells are responsible for propelling the tracheobronchial secretions towards the pharynx and are also active in transepithelial electrolyte transport. Goblet cells are responsible for the viscoelastic blanket of mucus that covers mucus of the bronchial epithelium (Mills *et al.*, 1999; Ganz, 2002). The ultimate role of bronchial epithelial (Figure 4) is the initiating and augmenting

pulmonary host defence mechanisms, both in health and in disease, by synthesizing and releasing a variety of mediators that can cause inflammatory cell differentiation, chemotaxis, and activation (Mills *et al.*, 1999).

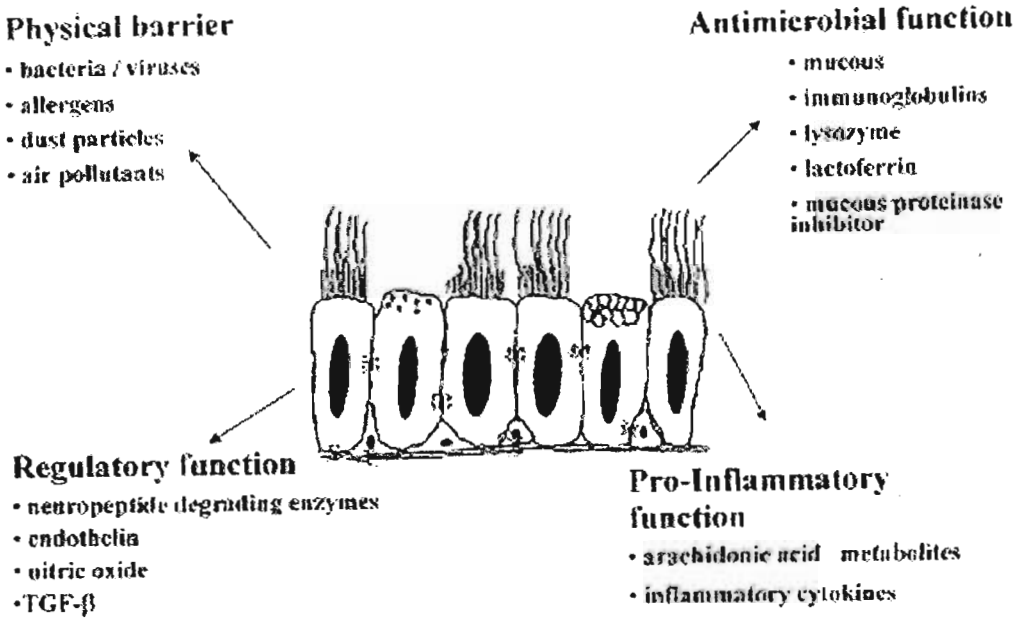

Figure 4: Multifunctional role of the airway epithelium in both health and diseased cells (Mills et al., *1999).*

In vitro studies have measured the responses of a bronchial epithelial cell line when induced with particles and their associated cytokines that are released after induction and resolution of inflammation (Holgate *et al.*, 2000; Veranth *et al.*, 2007). The ability of lung epithelial cell to secrete a range of mediators, such as interleukin-6 (IL-6) and IL-8 when induced with dust have been studied (Larsson *et al.*, 1997; Roespstorf & Sigsgaard, 1997, Palmberg *et al.*, 1998, Hansen *et al.*, 1999; Allermann *et al.*, 2002). Furthermore, cell wall damage was noticeable and measured by the release of LDH in A549 human epithelial and macrophage (THP-1, Mono Mac 6) when induced with ultra fine particles (Wottrich *et al.*, 2004). The elevated release of LDH was observed when ultra small particles were used, moreover, two macrophage behaved differently from each other, THP-1 which is sensitive to dust, and Mono Mac 6 which is insensitive. Damage in the cell wall triggered the release of cytokines (IL-6 and IL-8) and TNF-

α. The ability of bronchial epithelial cells to synthesize and release a wide range of mediators in culture media with or without stimulation have been confirmed by most researchers, and the mediators include granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF-α), interleukin-6 (IL-6) and IL-8 (Xing *et al.*, 1998, Fuji *et al.*, 2001, Lee *et al.*, 2004; Becker *et al.*, 2005).

1.3.3.3.1 Human lung carcinoma epithelial cell line (A549)

A549 is an epithelial-like human lung carcinoma growing adherently as monolayer (Kwon and George, 1999). The cell line was initiated by Giard, *et al* (1973) through explants culture of lung carcinomatous tissue from a 58-year-old Caucasian male. Furthermore, Stearns *et al.*, (2001) defined A549 as type II cell line which have distinguishing ultra-structural features (Figure 5), including cuboidal-like shape, extensive cytoplasm with rough endoplasmic reticulum, tight junctions, and lamellar bodies. Lieber, *et al.*, (1976) revealed that A549 cells could synthesize lecithin with a high percentage of de-saturated fatty acids utilizing the cytidine diphosphocholine pathway.

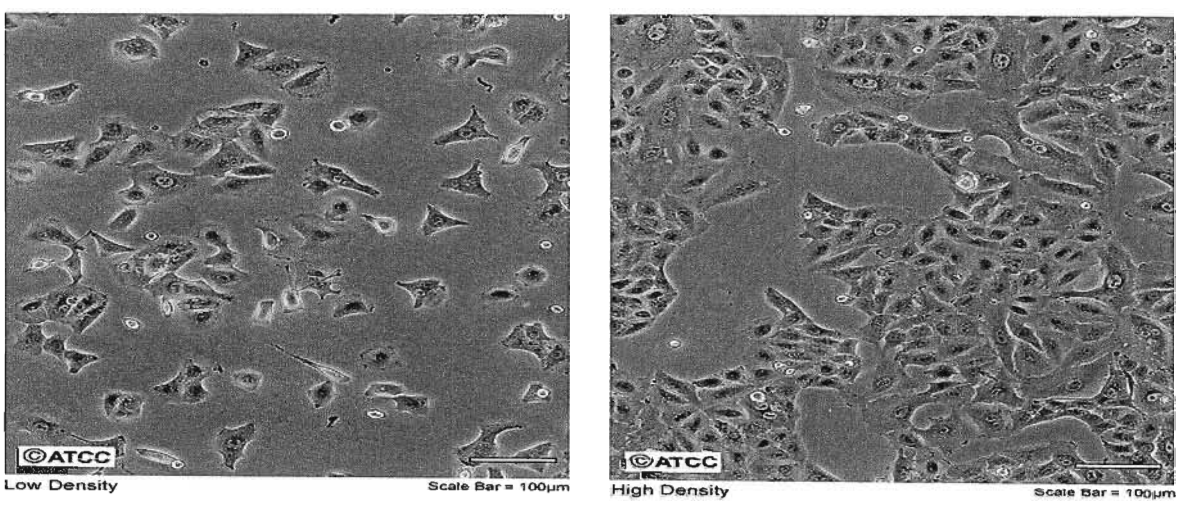

Figure 5: Morphology of A549 CCL-185 Human lung carcinoma epithelial cell line (Lieber et al., 1976).

Researchers focused on the A549 sensitization by ultra-fine particles due to the fact that it has a larger surface area that triggers pulmonary inflammation when infected by particles. Furthermore, A549 demonstrated the release of cytokines when sensitized with dust particles (Roepstorf and Sigsgaard, 1997; Burvall *et al.*, 2003). Stearns *et al.*, (2001) reported the ability of A549 to internalize ultra-fine particles, which were noted to be aggregated and predominately found in membrane bound vacuole or enmeshed in loosely or tightly bound lamellar bodies.

In other studies, Ramage *et al.*, (2006) investigated tobacco smoke effects on A549 to induce apoptosis. Tobacco smoke has been linked to alveolar epithelium damage. Tobacco smoke was shown to initiate apoptosis in airway epithelial cells, which were confirmed by the mitochondrial damages. Numerous studies have been published focusing on the A549 bioassay; and Deoxyribonucleic acid (DNA) fragmentation have been observed when cells are treated with foreign material such as dust particles (Roepstorf and Sigsgaard, 1997; Burvall *et al.*, 2002), toxic of tire debris extracts (Gualtieri *et al.*, 2005), and tobacco smoke (Ramage *et al.*, 2006). In addition, Cheng *et al.*, (2005) studied traditional Chinese herb extract (*Bupleurum scrozonerifolium*), and *in vitro* results showed the ability of herb extract to initiate apoptosis of A549. It was further observed that A549 released cytokines in response to the environmental particles that are triggered by pulmonary inflammation (Hansen *et al.*, 1999, Mills *et al.*, 1999; Wottrich *et al.*, 2004). It would be useful to investigate whether A549 undergoes any DNA destructions when treated with house dust extract.

Interestingly, Adam *et al.*, (2006) reported the ability of A549 to release cytokines (IL-8) when being induced by house dust mites. Furthermore, house dust mites have been reported for being major allergens that contribute to elevated levels of allergic disease in humans, such as bronchial asthma, perennial rhinitis and dermatitis (Asokananth *et al.*, 2002). Kauffman *et al.*, (2006) confirmed that A549 released cytokines (IL-6 and IL-8) after being induced with house dust mite (HDM) extracts. House dust mite extract has been shown to activate airway epithelial in two

ways, namely by protease-dependent activation and protease-independent activation pathways. Protease-depend activation results in morphological changes, cell-desquamation and production of pro-inflammatory cytokines whereas protease-independent activation further boosts production of pro-inflammatory cytokines, without affecting cell morphology (Kauffman *et al.*, 2006).

1.3.3.3.2 Human lung (bronchus); virus transformed epithelial cell line (BBM)

This cell line was derived from normal human lung bronchus epithelial cell line (BEAS-2B) that was transformed with B-myc/pSV2neo plasmid and constructed by ligating a BamH1/EcoR1 fragment of the c-myc gene from CA46 cells to a BamH1/EcoR1 fragment of the pSV2neo plasmid (Reddel *et al.*, 1989). Epithelial cells were isolated from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals (Reddel *et al.*, 1989). The literature is limited with respect to the use of BBM *in vitro* studies. It was, therefore, deemed necessary to review published data concerning *in vitro* studies of BEAS-2B since BBM was derived from it. According to our knowledge BBM is the first contact with house dust extracts. Bronchial epithelial cells play a crucial role in mediating the path physiology of numerous respiratory diseases, such as asthma, chronic obstruction pulmonary disease (COPD), pulmonary fibrosis and lung cancer (Shetty *et al.,* 2008).

The ability of BEAS-2B to release cytokines, when stimulated with various substances such as dust particles, allergens, and heavy metals, has been reported. Veranth *et al.* (2004) reported the release of IL-6 and IL-8 when BEAS-2B was pre-treated with soil dust particles of various sizes. However, TNF-α was not detected which is strange due to the fact that it has been reported to be involved in the regulation of both IL-6 and IL-8 (Cuncha *et al.*, 1992). Other *in vitro* studies reported the release of IL-8 and TNF-α when stimulated with sub-micrometer particles and moreover an increase in the release of TNF-α was noticed when stimulated with bacterial

endotoxin (Huang *et al.*, 2003). The release of the following cytokines was reported: TNF-α, GM-CSF, IL-4, IL-6, IL-8 and IL-13 when BEAS-2B was stimulated with dust mite allergens (King *et al.*, 1998; Lordan *et al.*, 2002). Veronesi *et al.*, (1999) demonstrated the ability of BEAS-2B to release cytokines (IL-6, IL-8 and TNF-α) when induced with residual old fly ash (ROFA). The ability of BEAS-2B to release the above-mentioned cytokines when stimulated with ROFA has been confirmed (Quay *et al.*, 1998). ROFA has triggered the biological effect of cytokines to induce inflammatory response in the lungs. Residual old fly ash has been termed as a good particulate source to explore the role played by transition metals in lung inflammation and it is mainly emitted by power plants and other industries that burn heavy metal oil and contains substantial levels of several transition metals (Carter *et al.*, 1997; Quay *et al.*, 1998).

1.4 Cytotoxicology

Cytotoxicology is the study of how substances affect cells in cultured laboratory environment. There are number of analyses that can be conducted in order to investigate cytotoxic effect of the toxicants on cells. These include Lactate dehydrogenase (LDH), tetrazolium salt (XTT), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) etc. LDH is released upon cell death which is noticeable with the rapture of the membrane; whilst, XTT and MTT are based on the reduction of the tetrazolium salt by metabolic active cells (López *et al.*, 2003; Yin *et al.*, 2004). Therefore, MTT commonly used by researchers and was selected in this study on the bases of instrument availability (ELISA plate reader) for analysis. They are sensitive and useful tools to determine whether any materials contain significant quantities of harmful components and their effect on cellular systems. Trypan blue exclusion method is used as a screening test for measuring viability cells, whereby cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the colouring agent (Freshney, 1995). The evaluation of late mitochondrial DNA damages can be achieved using annexin –V – FLUOS methods (Roepstorf and Sigsgaard, 1992; Martina and Clynes, 1993).

1.4.1 MTT

MTT assay is a colorimetric assay which was first described by Mosamann in 1983 and is generally used for the assessment of mammalian cell survival and proliferation (Gerlier and Thomasset, 1986). The assay is useful to detect living but not dead cells; the signal generated depends on the degree of activation of the cells. The principle is based on cleavage tetrazolium rings by viable cells (via mitochondrial dehydrogenase enzyme); the pale yellow MTT is transformed into dark blue formazan crystals which are largely impermeable to cell membranes thus resulting in its accumulation with healthy cells. The number of surviving cells is directly proportional to the level of the formazan product created, and the results can be read by multi-well scanning spectrophotometrically (570 nm messenger and 630 nm reference filters) in an ELISA plate reader (Roespstorf and Sigsgaard, 1997; Johanning *et al.*, 1998). MTT has been widely used *in vitro* studies, for cytotoxicity measurement of the human cell lines viability following induced with various substances, in understanding human disease such as cancerous cells - HL-60 treated with *Narcissus tazetta* var. chinensis extracts (Liu *et al.*, 2006), A549 treated with *Angelica sinensis* extract (Cheng *et al.*, 2004). Feline Fetus Lung cells (FFL) treated with *Stachybotrys chartarum* (Johanning *et al.*, 1998). A549 together with peripheral blood mononuclear cells (PBMCs) treated with heat emitted house dust (Mathiesen, 2003), and A549 and monkey kidney cells treated with organic dust extract in the occupational settings (Roepstorf and Sigsgaard, 1997).

1.4.2 Annexin-V-FUOS

Annexin-V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS). Annexin-V-FLUOS (green) serves as a fluorescent probe for apoptotic cells which do not bind to normal cells (Roche). It is widely used in the detection of the two cell death stages which is apoptosis and necrosis. Apoptosis is defined as the programmed cell death or it can be referred as a mechanism for removal of unwanted, aged or damaged cells (Koopman *et al.*, 1994; Roser *et*

al., 2001). Apoptotic cells induce membrane change as it translocation of the PS from inner cytoplasmic side of the plasma membrane to the outer leaflet of the membrane (van Engeland *et al.*, 1998).

In contrast to apoptosis, necrosis is premature cell death which does not produce any regular pattern of DNA or protein degradation and is characterized by cell swelling, dilation of mitochondria, and increase in plasma membrane permeability and disruption of cellular DNA (Oshimi *et al.*, 1996). This usually occurs when the DNA undergoes de-fragmentation and internal organelles are released as the cell lyses (Nishikawa *et al.*, 2003).

In labeling cells with annexin-v which has specific affinity with PS and apoptotic cells will be stained green. Whilst, necrotic cells resulted from the internal organelles being released and propidium iodide (red) will stain necrotic cells (Roser *et al.*, 2001). Thus the assay involves simultaneous staining with both annexin-V-FLUOS and propidium iodide. Apoptotic cells stain positive for both annexin-V- and necrotic cells stained positive for propidium iodide which stain the cellular DNA and the results are measured by flow cytometry (Pläsier *et al.*, 1999).

Annexin – V together with PI have been frequently used to discriminate necrotic and apoptotic cells. Researchers have successfully measured the apoptotic and necrotic effect in various cell lines when induced with different test substances. Roser *et al.*, (2001) treated HT-29 colon adenocarcinoma cells with DNA damaging agent, Nishikawa *et al.*, (2003) treated neck squamous cell carcinoma with adenovirus vector, acrolein (cigarette smoke component) has been observed to induced human cell line death pathway. Nardini *et al.*, (2002) and Sakurai *et al.*, (2001) have shown the potential to reduce INS-1 cells viability when treated with alloxan, a mild oxidant, in a diabetes mellitus study. Furthermore, Caspase 3/7, released in the final stage of cell death activities has been measured by Yin *et al.*, (2004) in A549 human lung bronchial epithelial cell line treated with *Scutellaria barbata* extract.

In this study it was deemed necessary to screen cell viability of epithelial cell lines using trypan blue exclusion method, MTT and Annexin –V- fluos assay to better understand the process of cell death, either apoptosis or necrosis that the cell undergoes when treated with foreign material, namely house dust extract. The viability markers are used in automated assays to measure cells at biochemical levels which only occur in living cells and stop after cell death. The limitation of such indicator dyes that undergo a change in physical properties as a result of biochemical events limited to living cells have proven to be useful as markers of cells viability (Riss and Moravec, 2004).

1. 5 Summary

In this chapter, the aims and objectives of the study together, with the review of related literature have been presented and discussed. The literature broadly focused on the indoor air pollutants such as biological, physical and chemical contaminants. In addition, toxicology test methods were reviewed briefly. Chapter two covers research methodology, and collection and management of data to meet the objectives of the study.

CHAPTER TWO - MATERIALS AND METHODS

2.1 Ethical approval

Ethical approval for this study was obtained from the University of KwaZulu-Natal's Biomedical Research Ethics Committee as part of the South Durban Health Study (SDHS) reference number E117/03 and the laboratory analysis H166/05.

2.2 Sample selection and collection

House dust samples were obtained from households that participated in the SDHS. Households (n=20) from seven (7) communities were recruited with a total number of (n=140). A total number of 130 households participated and 10 households declined to participate. The communities that participated were from the following Durban regions:

South: Lamontville, Merebank, Bluff, and Wentworth.

North: KwaMashu, Newlands East and Newlands West.

The samples were collected during the period of July 2004 to September 2005. A portable vacuum cleaner (Wap Combi Cleaner Vs 300s, Germany) with a specialised unit that collects dust into the filter paper with particle retention of 8 μm (Appendix A) was used. The collected samples were kept in polyethylene bags and refrigerated at 4°C prior to laboratory analysis (Jafta, 2007). The samples were collected from the following surface areas: living room couches (n=59), bed mattresses (n=39) and carpets (n=7). The surface areas of interest were chosen based on the time spent by children within selected areas, due to the fact that SDHS mainly focussed on children's exposure to pollutants. Two samples were missing and twenty three (n=23) were below 1 g and were excluded from the study as there was insufficient dust for laboratory analysis (more than 1g was required). Consequently, one hundred and five samples (n=105) were valid for the analyses. Outdoor samples were not obtained in this study.

2.3 Isolation and identification of moulds

2.3.1 Media and saline preparations

Dichoran-glycerol (DG18) agar (31.5g) was weighed and placed into a conical flask and distilled water was added to a final volume of 1000 ml. The mixture was well agitated and boiled to dissolve the agar, and 100 ml of AR grade glycerol was added into the agar base. Then 0.10 g of chloramphenicol selective supplement SR78 was added to inhibit any bacteria growth that might have been present in the sample (Basalan *et al.*, 2004). This was sterilised by autoclaving at 121°C for 15 minutes and the medium was allowed to cool (Hocking and Pitt, 1980), and there after it was aseptically poured out into sterile Petri dishes (90 mm diameter) and allowed to set.

Malt extract agar (MEA) (50g) was weighed and placed into a conical flask; distilled water was added to a final volume of 1000 ml and this was followed by a thorough mixing and boiling to dissolve the agar completely. The solution was sterilised by autoclaving at 115°C for 10 minutes and then allowed to cool (Onions *et al.*, 1981). The media were aseptically poured out into sterile Petri dishes (90 mm diameter) and allowed to set.

In the preparation of the phosphate buffer solution (PBS) one tablet was dissolved in distilled water in a conical flask, and transferred into a 1000 ml volumetric flask. In the solution, 10 ml of Tween 20 was added, and distilled water was added to create a volume of 1000 ml. The mixture was mixed properly and 9 ml was aliquoted into 10 ml bottles and sterilized by autoclaving at 121°C for 15 minutes.

2.3.2 Mould isolation and analysis

House dust sample of 200 mg was added to 10 ml of sterile saline solution and allowed to stand for 15 minutes. This was followed by shaking with rotor mixer for 2 hours. Serial dilutions were prepared by pipetting 1 ml of dust sample into a test tube 1 that contained 9 ml sterile saline solution (10^{-1}) and mixed with house dust sample with a vortex mixer and then 1ml was aseptically transferred into a test tube 2 containing 9 ml of sterile saline solution (10^{-2}) and vigorously mixed before 1 ml was drawn for next test tube. The procedure was done for 10^{-3}, and 10^{-4}, respectively. A new pipette was used for each dilution during the transfer of each sample between the test tubes from concentrated to most diluted sample. One millilitre (1 ml) aliquot was plated out from test tube (10^{-4}) onto a surface of DG18 medium and plates (90 mm diameter), swirled and labelled underside with the dilution factor. This was repeated for 10^{-3} and 10^{-2} (from the most diluted to the higher concentrated sample). Petri dishes were individually wrapped with parafilm around the edges of the plate to prevent cross-contamination and incubated at 25°C for 7 days. All the work and analysis was carried out in triplicate. The aseptic technique was adhered to at all times as to minimize or prevent any contamination. Mould analysis was done under the laminar flow cabinet (LABOTEC, SA). After the incubation period, mould colonies were visibly counted and expressed as colony forming units per gram (CFU/g) of house dust.

2.3.3 Cultivation of moulds for classification purpose

The recovered colonies (2.3.1.2) were inoculated onto the surface of MEA and incubated at 25°C for 14 days. Fungal slides were prepared by placing a small sample of fungal mycelium into a clean slide. The hyphae were teased out with dissecting needles until completely separated, and 1-2 drops of lacto-phenol blue (LPB) was added into the stain. The cover slip was placed over and viewed under a light microscope as per the method previously described by Smith, (1946).

The fungi colonies were classified on the basis of colony morphology and isolates were then identified to genus level under the light microscope. In addition mycology books were used for further classification (Smith, 1946, Onions *et al.*, 1981; Deacon, 1997). The isolated moulds that dominated in this study were sent to the mycology laboratory at Allerton Provincial Laboratories, Pietermaritzburg for further confirmation.

2.4 Analysis of heavy metals

House dust samples of 1 gram (n=105) were individually packed into polystyrene bags. The samples were chilled in ice pack during transportation to Umgeni Water Board, an independent accredited laboratory in Pietermaritzburg, where heavy metal analyses were carried out. The house dust extraction was done according to the extraction protocol for each heavy metal of interest. The in-house analytical quality control (AQC) and standard stock solutions were used for validation and reliability of the results. In summary, AQC samples are included in each set of analyses and the results of the real samples are only accepted if the value of the AQC fell within certain statistically calculated limits. Generally, an AQC is analysed at the beginning of a run, after every ten samples, and at the end of the run, unless otherwise stipulated in the method. In case of the stock solutions are prepared from certified analytical reagent (AR) grade chemicals with active ingredient purity, where possible, of 99% or better. The calibration of the instruments and standardization are prepared by trained personnel (technician) and certified by technical supervisor before samples are run for analysis.

2.4.1 Sample digestion and analysis

2.4.1.1 Mercury (Hg)

House dust was digested (as to release Hg) after weighing 0.0501 – 0.0668 g of house dust into 100 ml of ultra-pure water then 2 ml concentrated nitric acid and 1 ml potassium dichromate were added followed by the addition of 1 ml sulphuric acid and 5 ml potassium permanganate

into the beaker. The beaker was covered with a glass Petri dish and the sample was digested in a preheated water bath (set at 80±5°C) for at least 8 hours. The sample was removed from the water bath and cooled to room temperature, and 2.5 ml of bleach was added and mixed in order to decolorize excess potassium permanganate. The sample was then re-suspended in 150 ml of ultra-pure water. Mercury was analyzed by atomic absorption spectrometer with a 253.7nm mercury lamp and standardized methods were used (Appendix C).

2.4.1.2 Lead (Pb)

House dust was digested (as to release Pb) in the pre-weighed 0.0493 – 0.576 g of house dust suspected in 200 ml of ultra-pure water with added 2 ml concentrated nitric acid. The solution was brought to boil using a hot plate. The mixture was evaporated to approximately 100 ml and cooled in room temperature. The sample was re-suspended into 200 ml of the ultra-pure water and measured with certified measuring cylinder. Lead was analyzed using inductively coupled plasma spectrometer following standardized methods (Appendix D).

2.4.1.3 Arsenic (As)

House dust was digested (as to release As) by weighing 0.0483 – 0.6019 g of house dust sample into 30 ml of ultra-pure water, and 12 ml of hydrochloric acid was added into the mixture and brought to boil using the hotplate until the volume was approximately 20 ml. The beaker was removed from the hotplate and allowed to cool at room temperature and was then transferred into a certified measuring cylinder and the mixture was re-suspended with 30 ml ultra-pure water ultra-pure. The sample was further transferred from certified measuring cylinder into a beaker and 0.6 ml of potassium iodide solution was added and left to stand in room temperature for an hour before analyses. Arsenic was analyzed using standardized methods with the aid of a PS Analytical Automatic Hydride Generator (Appendix E).

2.5 Cytotoxicity testing

2.5.1 Dust extraction

The house dust sample was extracted according to allergen protocol (Indoor Bitechnologies, Charlottesville, USA). Briefly, house dust samples (100 mg) were each weighed and transferred into a 75 mm x 12 mm plastic test tube, and 2.0ml phosphate buffered saline with 0.05% Tween 20 (PBS-T) was added. The mixture was re-suspended in PBS-T and vigorously mixed with the aid of the vortex mixer (Vortex – Genie 2, Scientific Industries), followed by shaking for 2 hours on a laboratory rocker shaker (Stuart Roller Mixer SRT2, Barloworld Scientific) at room temperature. Sample was centrifuged at 2,500 rpm for 20 minutes at 4°C (Hermle Z 300K, LaborTechnik). Approximately 1.5ml of the supernatant was removed using a plastic Pasteur pipette and pellets (containing settled soil particles) were discarded. Supernatant was separated and stored in cryo vials at -20°C freezer with sample number and date clearly labelled prior to further laboratory analysis. Allergen is the final product that was suspended and has been reported to be prevalent indoor households (Platt-Mills, 2007).

2.5.2 Cell proliferation

2.5.2.1 Human bronchial lung carcinoma epithelial cell line - A549

The human lung epithelial cell line A549 (ATCC No. 185-CCL) was obtained from Medical Microbiology Laboratory University of KwaZulu-Natal School of Medicine after 49 passages. Cells were revived in Eagles minimum essential medium (EMEM) since it was frozen in it and supplemented with 10% foetal bovine serum (FBS) that was heat-inactivated in the water bath at 56°C for 30 minutes according to manufacture's instructions (Cambrex/Lonza, Basel - Switzerland). Frozen cells were retrieved from -80°C ultra freezer and immediately thawed and seeded onto 75 cm^2 tissue culture plastic flask and grown overnight in humidified 5% CO_2 incubator at 37°C. After the incubation period the cells were washed twice with Hank's

Balanced Salt Solution (HBBS) and the medium was changed into 50% EMEM and 50% Ham's F-12, which was supplemented with L-glutamine together with 5% FBS and 1 % non-essential amino acid (NEAA). Cells were further incubated in 5% CO_2 at 37°C for 24 hours, and medium was changed every second day until confluence growth of 80 – 90% was reached, which was noticed to be after 72 hours with the combination of the two media (EMEM and Ham's F-12) and thereafter, cells were trypsined as described below in 2.5.2.3.

2.5.2.2 Human lung (bronchus) virus transformed epithelial cell line - BBM

The human lung epithelial cell line BBM (ATCC No. 9482 - CRL), was obtained from Medical Microbiology Laboratory University of KwaZulu-Natal School of Medicine after 54 passages. Cells were revived in a coated 25 cm^2 tissue culture flask (coated with 1% albumin from bovine serum minimum 98%) and grown in bronchial epithelial growth medium (BEGM) bullet kit growth medium, which was supplemented with 2 ml bovine pituitary extract (BPE), and other aliquots of 0.5 ml each namely: - hydrocortisone, epidermal growth factor (hEGF), epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B and retinoic acid, in 500 ml media bottle (Cat. No. CC-3170, Cambrex/Lonza, Basel – Switzerland). BBM cells were grown overnight in a humidified 5% at 37°C CO_2 incubator. After the incubation period, cells were washed twice with HEPES – buffered saline solution (HEPES-BSS) and the media were changed. Cells were further incubated for 24 hours in humidified 5% CO_2 incubator at 37°C, and media was changed every second day until confluence growth of 80 – 90% was reached thereafter the cells were trypsined as described below.

2.5.2.3 Trypsinisation

Once the confluent growth of approximately 80 -90% was reached the monolayer of cells was washed twice with HBSS (A549) and HEPES-BSS (BBM) to remove unattached cells which

might have inhibited the action of the enzyme (tyrpsin). Cells were treated with 0.25% trypsin–versene, and incubated in a humidified 5% CO_2 incubator at 37°C for approximately 10 minutes to detach cells. Once cells were detached the reaction was neutralised to stop the action of tyrpsin by the addition of 0.25% of FBS. Cells were transferred into the new flask with a bit more cells suspension in it, and freshly prepared media with 10% FBS for A549 and BEGM media for BBM was added into flasks and incubated as described above. Cell suspension of 0.25% was aliquoted into 2.0 ml graduated micro-centrifuge tube (Cat No. 508-GRD, Cambrex/Lonza) for viability checking as described below in 2.5.3.4.

2.5.2.4 Trypan blue exclusion

The trypan blue dye exclusion method was used to determine cell viability. The viable cells do not take up the stain due to their intact membrane and appear colourless, whilst, dead cells are permeable to the stain and appear blue. Briefly, the surface of the haemocytometer and cover-slip was cleaned with 70% (w/v) ethanol and dried. After trypsinisation, a cell count was done by adding 10 µl of cell suspension into 100 µl of trypan blue dye together with 100 µl of phosphate buffer (PBS). The mixture was gently mixed by pippeting up and down before 10 µl samples was drawn and mounted onto the haemocytometer. Cell count was done under a light microscope at 40x magnification, which was done each time before cells were seeded either in tissue culture flasks or in 24 and 96 well plates.

The number of cells per ml was calculated as follows:

$$\frac{N \times y \times 10^4}{4}$$

Where: N = total number of cells counted in 5 chamber squares

10^4 = correction factor for volume (ml) over each large square

y = dilution factor

4 = number of chambers counted

2.5.2.5 Cyropreservation of cells

Once confluent growth was reached cells were washed twice with HBSS (A549) and HEPES-BSS (BBM), and trypsinised as described in 2.5.2.3. Cells were recovered by centrifugation at 400 rpm for 5 minutes in 4°C (Multifuge 3S-R, Heraeus) and supernatant was discarded. Cell pellets were re-suspended in 1000 µl of freezing fluid which was prepared as follows: 60% growth media, 20% FBS and 20% dimethyl sulfoxide (DMSO) for A549, and L-15 and BEGM supplemented with 2 mM L-glutamine, 10% FBS and 7.5% DMSO for BBM. The procedure was conducted with ultra care while adding DMSO drop-wise, due to its detrimental effects on cells. Approximately 1.5 ml was aseptically aliquoted into cryo vials and was subjected to slow freezing (vials was placed in polystyrene and stored over night at -70) before being transferred into to -80°C for long term storage with clearly labelled passage number and date.

2.5.2.6 Ethanol cell fixation

After trypsinazation, cells were recovered via centrifugation at 400 rpm for 5 minutes. The supernatant was discarded and pellets were washed once with phosphate buffer (PBS) and re-suspended in 1000 µl PBS prior to fixing with ice cold ethanol. Ice cold ethanol (1000 µl) was added drop wise while vigorously mixing cells to avoid any clumping during the fixation process for approximately ≥1 hour, 4°C in ice (Uhal *et al.*, 1998). Thereafter, samples were refrigerated at 4°C until all samples (n=105) were treated, which took approximately 4 weeks, due to the number of samples (n=20) that could be processed at a given time. Cell lines were treated with house dust extract as per assay conducted and analysed once all the batch in a particular cell line were completed (A549 and BBM).

2.5.3 Bioassays

2.5.3.1 MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)

Cells (A549 and BBM) were grown in 25 cm^2 tissue culture flask as described in 2.5.2. Once confluence growth (80-90%) was reached cells were trypsinized as described in *2.5.2.3* and 1.0 x 10^5 cells/ml was seeded into 32 ml growth media (EMEM, Hams F-12 and 10% FBS) for A549 and BEGM media for BBM. The MTT assay was conducted according to the manufacture's instructions: constant volumes of cells (100 µl) was seeded into each 96-well microtitre plate and incubated in a humidified 5% CO_2 incubator at 37°C for 24 hours. After the incubation period, 100 µl of house dust extracts as per dilutions (1-100 µl) were added into 96 wells as indicated in Table 2 below and incubated overnight in a humidified 5% CO_2 incubator at 37°C. After the incubation period, medium was removed from the wells and replaced with 10 µl solution of tetrazolium salt, in addition to this 100 µl of fresh growth media were added and incubated for a further 4 hours in 5% humidified CO_2 incubator at 37°C (Cat. No. 11 465 007 001, Roche; Appendix F). Following this, 100 µl solubilization solutions were added and plates were further incubated overnight in a humidified 5% CO_2 at 37°C. Cell mortality was analysed using an enzyme-linked immunosorbent assay (ELISA) plate reader at 550 – 600 nm (Bio-RAD, 3550 Micro plate reader). The negative (untreated cells) controls were subjected to the same environment (such as incubation temperature) as test samples and analyzed in triplicate in line with test substance (house dust extract).

Table 2: House dust extracts dilution and human cell lines.

HOUSE DUST EXTRACT	MEDIA W/O CELLS	CELLS (1.0×10^5 cells/ml)	FINAL DILUTION
100	0	100	1:2
50	50	100	1:4
20	80	100	1:10
5	95	100	1:40
1	99	100	1:200

2 5.3.2 Annexin -V- Fluos Stain

2.5.3.2.1 Analysis by fluorescence microscopy

A549 cells were grown in 75 cm^2 tissue culture flasks and BBM were grown in a coated 25 cm^2 tissue culture flask and once confluent growth was reached cells were trypinized as described here above (2.5.2.3). Constant volume of cells (1000 µl) were seeded (1.0×10^5 cells/ml) in each 24 well tissue culture plates that were already pre-inserted with sterile round cover slips (12 mm x 1.5 thickness) (Cat no. 72196-12, SMM Instruments), to accommodate cell attachment and incubated overnight in an humidified 5% CO_2 incubator at 37°C. After the incubation period, cells were treated with house dust extracts using 1:10 dilution; and the untreated cells were used as control and cells incubated overnight in a humidified 5% CO_2 incubator at 37°C. After the incubation period, media were removed and cells were washed once with PBS and fixed with ice cold 70% ethanol as described above in 2.5.2.6. However, plates were not centrifuged as described in the fixation procedures and they were kept in the refrigerator for 3 weeks prior to staining. Cells were retrieved from the refrigerator and washed once with PBS, which was followed by staining with 20 µl of annexin - V and 20 µl of PI for 15 minutes which was mixed with 1 ml incubation buffer (Appendix G) which was sufficient for 10 samples (Cat. No. 11 828 681 001, Roche: Appendix H). Round cover slips were taken out of 24 well plates with the aid of sterile needle and forceps and allowed to dry in dark room temperature. Slides were gently dried with a paper towel by blotting excess dye without removing cells that were attached on the

round cover slips. Once the cells were completely dried they were mounted with p-xylene-bis-pyridinium bromide (DPX) in glass slides and analyzed with fluorescence microscopy at 515 – 565 nm (green). Stains are sensitive to light and this procedure was done in the dark and the slides were wrapped with foil before and after reading.

2.5.3.2.2 Analysis by flow cytometry

A549 cells were grown in 75 cm^2 tissue culture flasks, and BBM were grown in a coated 25 cm^2 tissue culture flask. When confluent growth was reached cells were treated with dust extracts using 1:10 dilution and A549 was incubated overnight in a humidified 5% CO_2 incubator at 37°C; BBM was only incubated for 6 hours. After the incubation period, cells was washed twice with PBS and trypsinized as described previously. Cells were transferred into 5 ml falcon tubes and centrifuged at 2500 rpm for 5 minutes at 4°C, and thereafter washed once with PBS and re-suspended in 1000 µl PBS. Concurrently, positive controls were conducted in parallel with samples for both negative (unstained cells) and positive as tabulated in Table 3 below. The positive control used for A549 was actinomycin D (CAS No. 50-76-0, Sigma: Appendix I), and for BBM was cycloheximde (Cas No. 66-81-9, Sigma: Appendix J). Actinomycin D (AD) has been previously described as an antineoplastic antibiotic that inhibits cell proliferation. Its ability to induce apoptosis against tumour cells has been reported by Watanabe *et al.*, (2002), Lecoeur *et al.*, (2002), Rogatsky *et al.*, (2003); McCann and Imani, (2007). Cycloheximide (CHX) is a glutarimide antibiotic derived from a microbial source. It has been used in normal bronchus cell line to determine apoptotic effects on treated cell and it has been shown to block the translation of messenger RNA in the cytosol (Arima *et al.*, 1999; Wadsworth *et al.*, 2003).
Cells were fixed with 70% ice cold ethanol as described above in 2.5.2.6. When all samples were treated as described here above, they were retrieved from the refrigerator and separated by centrifugation at 2500 rpm for 5 minutes at 4°C (Multifuge 3 S/3R, Heraeus) prior to staining. Cells were stained with 20 µl annexin-V and 20 µl PI solutions which were mixed with 1 ml

incubation buffer, which is enough for 10 samples, and its has been described in the manufacturer's protocol (Cat. No. 11 828 681 001, Roche: Appendix H). The controls were also stained as tabulated in Table 3. Controls and cells were analysed by flow cytometry (LSR II, BD Science) at 488 – 617 nm within an hour after staining. The double staining (annexin V and PI) allowed the detection and differentiation of cells, apoptotic (annexin positive and PI negative), and necrotic cells (double positive). The apoptosis and necrosis percentage was calculated using the following equation as previously described by Ishii *et al.*, (2004).

$$\text{Apoptosis (\%)} = \frac{\text{Number of [Annexin V (+) and PI (-)] cells x 100(\%)}}{\text{Number of PI (-) cells}}$$

$$\text{Necrosis (\%)} = \frac{\text{Number of PI (+) cells x 100 (\%)}}{\text{Number of all cells}}$$

Table 3: Controls for flow cytometry analysis.

CONTROLS	**STAINS**
Positive control(s): - BBM (CHX) and A549 (AD)	Annexin – V – and PI
Negative control – untreated cells	Unstained cells
Necrosis testing	PI
Apoptosis testing	Annexin – V -

2.5.3.3 Human cytokines analysis

2.5.3.3.1 Cell preparation

Human cell lines (A549 and BBM) were propagated as described above in 2.5.2, and treated with house dust extract, as elaborated in 2.5.3.2, and refrigerated at 4°C, after house dust extract were preserved in 70% (w/v) ice cold ethanol described in 2.5.2.6. Cells were retrieved from the refrigerator and separated from 70% ethanol by spinning at 2500 rpm for 5 minutes at 4°C and thereafter washed once with PBS. A549 was re-suspended in 1000 µl of growth medium (Ham's F-12, EMEM and 10% FBS), and BBM was re-suspended in 1000 µl of and both cells were stored in -80°C for three weeks then stored into liquid nitrogen during transportation to Microbionix- Institut für Medizinische Mikrobiologie und Hygiene in German for multiplex human cytokines analysis.

2.5.3.3.2 Luminex®100 cytokines detection

House dust extract was thawed at room temperature and centrifuged at 14,000 rpm for 10 minutes and filtered prior to analysis to prevent clogging of the filter plates. Human cytokines Luminex®100 custom 5-plex kits (for IL-4, IL-6, IL-8, IL-13 and TNF-α) were analysed at Microbionix (Germany). All reagents were purchased from Biosource (Cat no. LHB0001) and prepared according to the manufacturer's protocol. The assays were performed in a 96 well filtered bottom plate according to manufacture's protocol. Antibody conjugated beads that were used provided at 10x concentrations which were diluted at 2.5 µl of 10x beads to make 1 stock solution, 25 µl per well requiring 30 seconds vortex followed by 30 seconds sonication in water bath to prevent bead aggregation. Lyophilized standards were reconstituted by serially dilution and diluents assay (50%) and cell growth medium (50%). This was undertaken to generate 7 standard concentration sets and diluent alone was used as the blank. The wells were pre-wetted with 200 µl working wash solution, for 30 seconds; and working wash solution was aspirated

with vacuum manifold with the bottom of the plate blotted on paper towel to remove excess fluid. The bead solution was vortexed for 30 seconds then sonicated for 30 seconds immediately prior to the addition of 25 µl diluted bead solution into each well. Once the beads were added into the wells, the plates were protected from light. The plate was then washed twice with 200 µl of working wash solution, soaked for 30 seconds and then aspirated using a vacuum manifold with the bottom of the plate blotted on paper towels to remove excess fluid. The incubation buffer was added (50 µl) in each well; and for the wells assigned to the standard curve 100 µl of standard dilution was added in each well. The wells assigned to the house dust extract, 70 µl assay diluents were added followed by 70 µl house dust extract. The plates were incubated for 2 hours in room temperature on an orbital shaker (500-600 rpm). After the incubation period, liquid was removed from the wells by aspirating with vacuum manifold followed by addition of 200 µl working wash solution to the wells. Thereafter plates was washed twice with 200 µl of working washed solution, which was soaked for 30 seconds and then aspirated using a vacuum manifold with the bottom of the plate blotted on paper towel to remove residual liquid.

Biontinylated detector antibody (100 µl) was added in each well and incubated for 1 hour at room temperature on an orbital shaker (500-600 rpm). Then the plate was washed twice prior to the addition of streptavidin-RPE (100 µl) into each well. The plate was further incubated for 30 minutes at room temperature on an orbital shaker. Finally, the plate was washed three times as described above. Working wash solution (100 µl) was added in each well and the plate incubated for 2-3 minutes at room temperature on an orbital shaker to re-suspend beads. The plate was uncovered and measured on the Luminex®100 instrument, and analysed using Liquichip-software version 1.0 from Qiagen. The analysis software was set up to acquire 100 events per single bead set. The raw data were measured as mean fluorescence intensity (MFI) and the concentration of the analyte in each sample was calculated using a 4 or 5-parameter logistic fit-curve generated for each analyte from the 7 standards. The lower limit of

quantification (LLOQ) was determined using the lowest standard that was at least 3 times above background. Calculation of the LLOQ was performed by subtracting the MFI of the background (diluent) from the MFI of the lowest standard concentration and back-calculating the concentration from the standard curve. The principle behind Luminex®100 analysis and interpretation has been described by Heijmans-Antonissen *et al.*, (2005), Szodoray *et al.*, (2007); Chowdhury *et al.*, (2009).

2.6 Statically analysis

The data was analysed using software package of Microsoft Excel 2000 and SPSS for Windows (15.1). Non-parametric test (Dunn's multiple comparison tests) were computed using Graph Pad Instant and ($p<0.05$) was considered to be statistically significant and ($p<0.01$) was considered highly significant.

2.7 Summary

This chapter covered research methodology, data collection and sample handling in terms of performing relevant laboratory tests to meet the objectives of this study. The results are presented in chapter three.

CHAPTER THREE - RESULTS

3.1 Mould analysis

House dust samples (n=105) from the Durban residential buildings were analysed in triplicate for culturable mould. There were (n=128) mould types that were isolated but only (n=105) moulds were identified. The isolated moulds are given in ascending order and the number of occurrences per surface areas: living room couches (56.2%)>bed mattresses (37.1%)>carpets (6.7%). The least moulds were recovered from carpets and possible explanations is that not all households that participated in the study were carpeted hence less samples were collected within this surface area.

In addition different type of genera that were isolated and identified as presented in Table 4. The predominant moulds were sent to Allerton Provincial Laboratory in Pietermartizburg for further verification and 75% of mould matched what has been reported in this study but 25% was not identified due to contamination; the results are shown in Appendix B. The predominant genera that were isolated and identified in this study from three surface areas mentioned here above are depicted in Figure 6. The mould species as per surface areas namely: - living room couches: *Rhizopus spp* (n=10), *Alternaria spp* (n=5), *Penicillium spp* (n=5), *Aspergillus* spp (n=4); bed mattresses: *Rhizopus spp* (n=4), *Aspergillus spp* (n=3) and carpets: *Penicillium* spp (n=2), *Spondonema spp* (n=2). In all the analysed household (south and north of Durban) samples, there was not a single sample that was free of moulds. In this study moulds were noticeable to be widely spread out in the communities isolated from.

Table 4: Households dust samples (n=105) from surface areas (living room couches, bed mattresses, and carpets). The frequencies of genera isolated are in brackets.

SURFACE AREA	GENERA	HOUSEHOLDS	PERCENTAGE
Living room couches	*Alternaria* (n=5) *Aspergillus* (n=10) *Byssochlamys* *Cladosporium* (n=2) *Coccidiodes* *Curvularia* (n=3) *Drechslera* *Fusarium* (n=2) *Geotrichum* (n=2) *Gliocladium* *Helminthosporium* *Modurella* *Moniliella* *Mortieralla* *Mucor* *Penicillium* (n=16) *Rhizopus* (n= 10)	59	56.2
Bed mattress	*Absidia* *Acremonium* (n=2) *Alternaria* *Aspergillus* (n=6) *Chaetomium* *Cladosporium* *Curvularia* *Drechslera* *Fusarium* (n=3) *Geotrichum* (n=2) *Helminthosporium* *Mortierella* *Mucor* (n=2) *Myceliphthora* *Penicillium* (n=6) *Rhizopus* (n=4) *Spondenema* *Trichoderma* (n=2) *Ulocladium* (n=2)	39	37.1
Carpets	*Myceliphthora* *Penicillium (n=2)* *Phialophora* *Rhizopus* *Spondonema (n=2)*	7	6.7

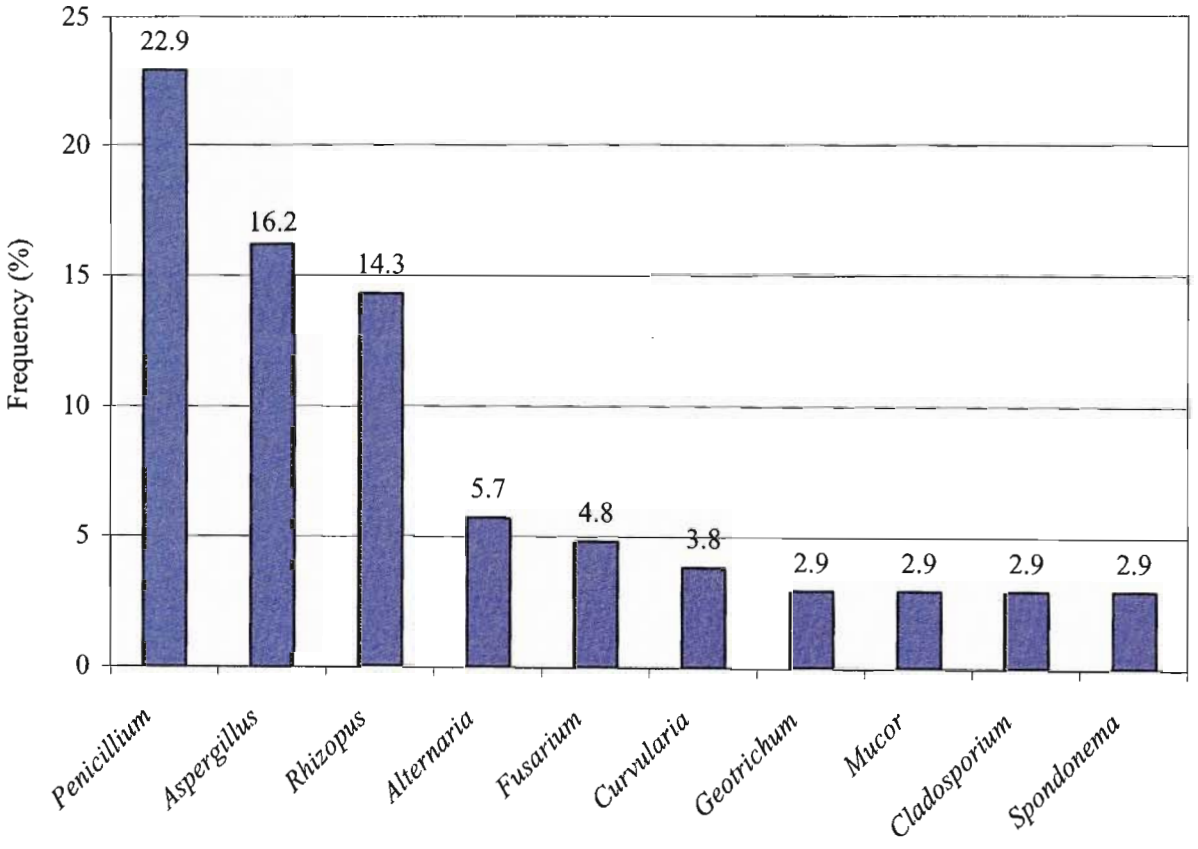

Figure 6: Predominant identified mould genera (n=10) that were isolated from Durban residential houses (south and north) in dust sample.

Mould incidences (n=105) were classified into mean *versus* genera (n=25) and the figure not shown. The isolated genera were as follows: *Absidia, Acremonium, Alternaria, Aspergillus, Byssochlamys, Chaetomim, Cladosporium, Coccidiodes, Curvularia, Drechslera, Fusarium, Geotrichum, Gliocladium, Helminthosporium, Modurella, Moniliella, Mortierella, Mucor, Myceliphthora, Penecillium, Phialophora, Rhizopus, Spondonema, Trichoderma,* and *Ulocladium*. The concentrations of identified genera ranged from 250 – 640 000 CFU/g.

The distribution of analysed moulds using the Box and Whisker's plot (Figure 7) indicates that the carpets' CFU/g are marginally scattered with values lies within 1.5 times the median. It is slightly similar to bed mattresses apart from the 25% values that lie below the median value. The distinction was observed in living room couches as the median is widely distributed and most of CFU values fall greater than 1.5 times median and do not lie within 99% of the median. Irrespective of the insignificant number of moulds recovered from carpets and more so for their ability to influence (p-value); and the isolates *Penicillium spp* ($p<0.323$) and *Rhizopus spp*($p<0.674$) were widely distributed throughout all three surface areas in greater proportions. Moreover, there were top three genera, which include *Aspergillus* that were dominant in this study. The overall highest mean reported in this study for *Penicillium* ranged from (3400 – 62316 CFU/g) recovered from living room couches followed by *Rhizopus* (5200 – 15990 CFU/g). The presence of mould indoor is a health risk for dwellers, which may range to allergic and toxins poising to those come in direct or indirect contact with spores.

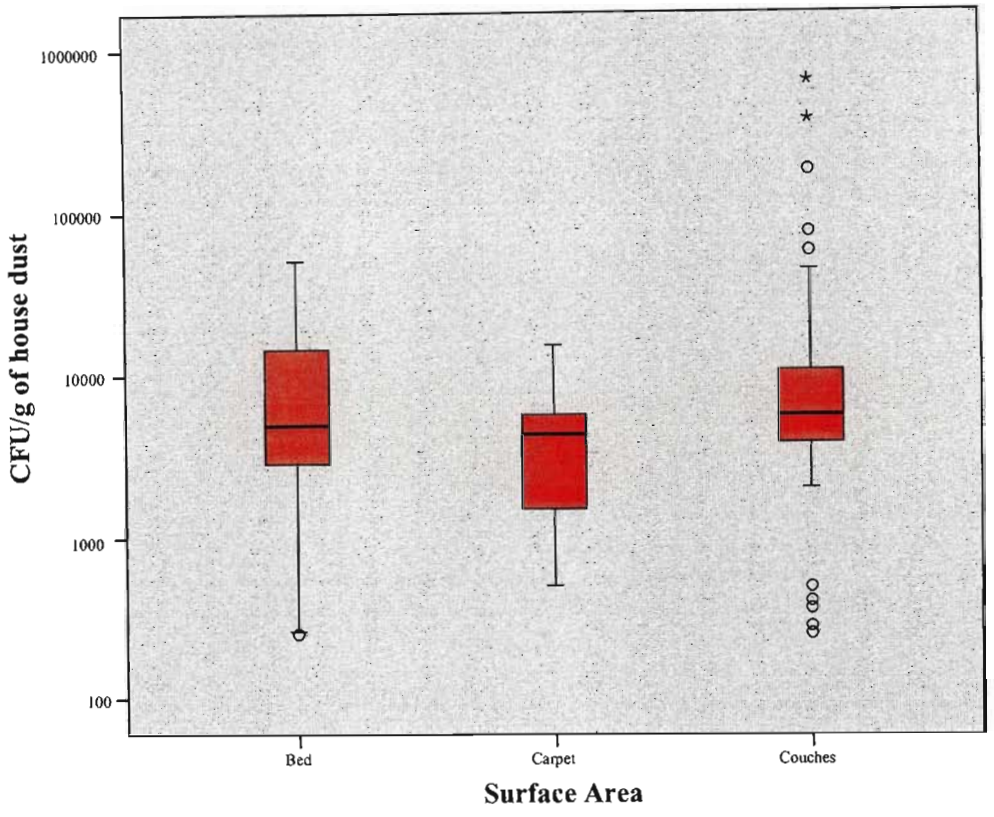

Figure 7: Box plot showing median concentration (in CFU/g of house dust) and distribution of total moulds by location. The horizontal line in each box is the median value thus the bottom of the box is 25th percentile and the top of the box is 75th percentile, respectively; the line extended above the box marks 1.5 median values. Circles, values, asterisk, values greater than 1.5 times the median value.

The number of CFU was compared with risk category guidelines compiled by Doe (2003) in Table 5. Risk category can be interpreted as follows: low (10,000 or less), moderate (10,000 – 100,000), high (100.000 – 1,000.000) and extremely high (1,000.000 or more). In this study, only two genera, namely *Aspergillus* and *Penicillium*, fall within the high risk category and were amongst the top three identified mould which includes *Rhizopus*. The results were below that reported by South African Occupational Health and Safety Act 85 of 1993 as amended, which

states that moulds above 1,000,000 CFU/g may be an indicator of the presence of unusual levels of moulds. However, most of the isolated moulds (for both south and north communities) were within OHSA ranges despite the ones that were under the 'high risk' category using the Doe (2003) classification.

Table 5: Mould genera that were within moderate and high risk category guidelines are represented in colony forming units of house dust per gram (CFU/g) inside the Durban houses sample.

SAMPLE AREA	GENERA	MOULD TOTAL COUNTS (CFU/g)	RISK CATEGORY
Couches	*Aspergillus*	27,000 – 370,000	High
	Penicillium	12,000 – 640, 000	High
	Rhizopus	44,000 – 75,000	Moderate
	Gliocladium	21,000	Moderate
	Modurella	18,000	Moderate
Bed mattresses	*Aspergillus*	17,000 – 39,000	Moderate
	Penicillium	12,000 – 50,000	Moderate
	Trichoderma	37,000	Moderate
	Spondenema	31,000	Moderate
	Cladosporium	26,000	Moderate
	Ulocladium	24,000	Moderate
	Fusarium	20,000	Moderate
Carpets	*Phialophora*	15, 000	Moderate

Low: 10,000 or less Moderate: 10,000 – 100,000 High: 100,000 – 1,000.000

Extremely High: 1,000.000 or more Doe (2003).

3.2 Heavy metals

Settled house dust samples (n=105) were collected from Durban residential buildings and analysed for heavy metals (lead {Pb}, arsenic {As}, and mercury {Hg}) at the Umgeni Water Laboratories in Pietermaritzburg. The summary of heavy metals results analyzed (Table 6), with the mean in ascending order namely: Pb (171.7 µg/g), As (4.3 µg/g), and Hg (2.2 µg/g). The Pb and As standard deviations were marginally spread apart indicated by results with mean which was fairly diverse and whereas Hg is within the mean. Pb was reported being the highest in Newlands West with ranges of 28.0-872.0 µg/g.

Table 6: The summary results of lead (Pb), arsenic (As), and mercury (Hg) in house dust of participating households.

	Pb	**As**	**Hg**
No. of homes sampled	105.0	105.0	105.0
Mean (SD) µg/g	171.7(21.5)	4.3(2.5)	2.2(2.3)
Range (µg/g)	28.0-872.0	1.3-18.4	0.6-19.0

Table 7 presents the result of heavy metals measured from communities (south and north of Durban and regional comparisons. The result of heavy metal (Pb, As and Hg) are presented in ascending order for the four communities in the south of Durban: Bluff followed by Wentworth, Merebank, and Lamontville. Exception was noticed for As and Hg with high levels in Merebank than other communities in the study. The same results presented in this table for the three communities in north Durban are given in ascending order: Newlands West, Newlands East, KwaMashu, apart from Newlands East with respect to As being higher than KwaMashu. The regional comparisons of heavy metals in south and north communities are also included in this

table. South Durban showed mean highly significant difference ($p<0.01$) in Pb concentration compared to the North significant difference ($p<0.05$) in mean As concentration and no significant difference in the mean Hg concentration.

Table 7: The heavy metals in house dust from the communities (south and north of Durban) and regional comparisons.

Location	Pb Mean (SD) Range (µg/g)	As Mean (SD) Range (µg/g)	Hg Mean (SD) Range (µg/g)
South region (n=51)	229.3 (93.5)** 102.0-542.0	4.8 (2.8)* 1.5-18.4	1.9 (1.2) 0.6-6.6
Merebank (n=20)	255.2 (108.0) 109.0-542.0	5.17 (4.1) 1.5-18.4	2.1 (1.5) 0.9-6.6
Lamontville (n=18)	174.6 (64.9) 102.0-345.0	4.1 (1.7) 2.2-9.0	1.5 (0.9) 0.6-3.3
Wentworth (n=8)	261.9 (73.1) 167.0-380.0	5.2 (1.9) 3.2-9.1	1.7 (1.1) 0.9-4.2
Bluff (n=5)	270.6 (74.5) 200.0-376.0	5.2 (2.9) 2.8-10.0	2.4 (0.8) 1.6-3.4
North region (n=54)	117.2 (12.5)** 28.0-872.0	3.7 (1.9)* 1.3-9.2	2.6 (2.9) 0.9-19.0
Newlands West (n=19)	177.9 (186.2) 28.0-872.0	4.2 (2.4) 1.3-9.2	3.7 (4.1) 0.9-19.0
Newlands East (n=19)	81.2 (29.9) 42.0-140.0	4.2 (1.8) 1.7-8.2	1.6 (0.9) 1.0-4.4
KwaMashu (n=15)	88.9 (37.9) 38.0-169.0	2.7 (0.7) 1.8-4.2	2.4 (2.5) 0.9-7.7

Statically significant differences between the two regions: - **$p<0.01$ (highly significant) and *$p<0.05$ (significant).

3.3 Cytotoxicity testing

The cellular activities were assessed using house dust extract as has been mentioned above in the methods and materials sections, not the actual house dust.

3.3.1 MTT assay

The toxic effect of house dust extract was analysed with MTT assay and a dose response curved was plotted (Figure 8) for both cell lines, and the mean and standard deviation were computed. The cell viability before the experiment was slightly above 80% in both cell lines. The results showed that cell viability for both cell lines went below 50% following exposure to various dilutions of house dusts extracts as reflected in methods and materials. It can be interpreted that house dust extract is highly toxic to bronchial epithelial cells with reduced cell viability for A549 (<40%) and BBM (<50%) cells in all dilutions used. The suitable dilution (1:10) was selected for further analysis in this study due to the fact that both cell lines are congruent at this point. Furthermore, house dust was noticeable to be responsible in lowering cell viability.

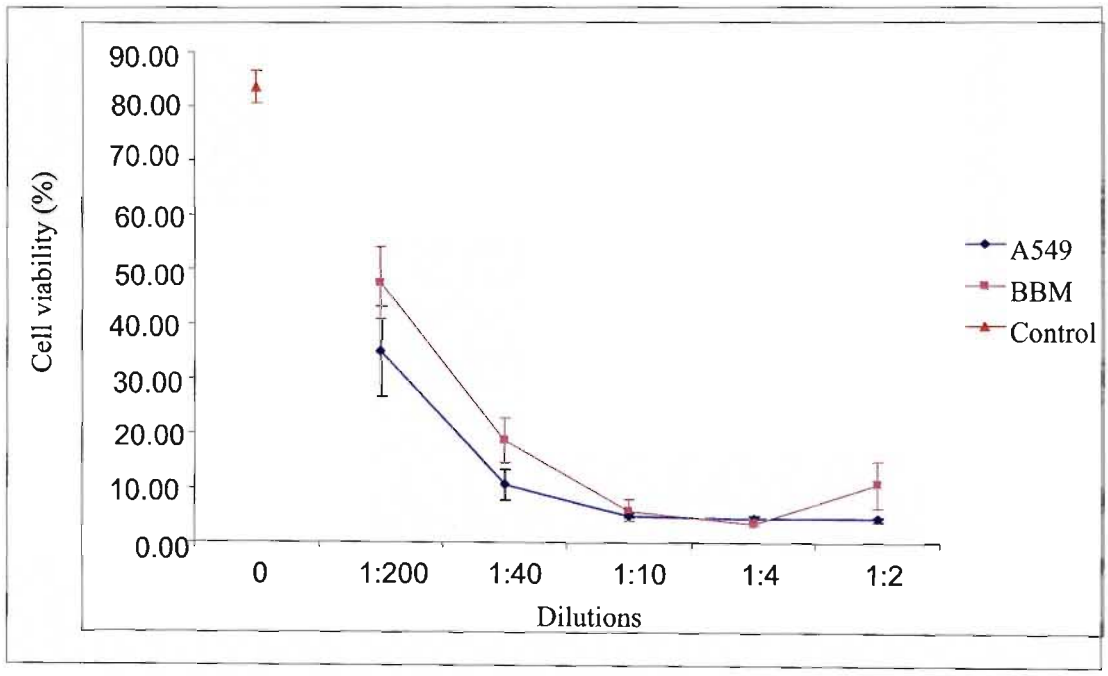

Figure 8: MTT dose response curve for A549 and BBM cell viability with different dilutions and represented in mean and 95% CI.

3.3.2 Determination of apoptosis and necrosis

Human cell lines that were exposed to house dust extract (1:10) dilutions for 12 hours were analysed for apoptosis and necrosis with flow cytometry. The percentage summary of both apoptotic and necrotic cells are shown in Figure 9 and it can be seen that median value for both cells lies just below 90% in Diagram A. The media was used instead of mean and the reason being results were not normally distributed (skewed). The percentage distribution for necrosis in both cells varies with the A549 median value below 30% while the BBM median value is just above 80%. Non-parametric test was done for human cell lines and were non-significant for apoptosis A549 versus BBM ($p<0.039$); while necrosis were noted to be significant ($p<0.000$) for BBM versus A549.

The positive controls for A549 (AD) are presented Figure 10 and in Figure 11 for BBM (CHX). It was observed that both known agent induced cell death with 97% necrosis of the BBM cell line and 93% apoptosis of the A549 cell line.

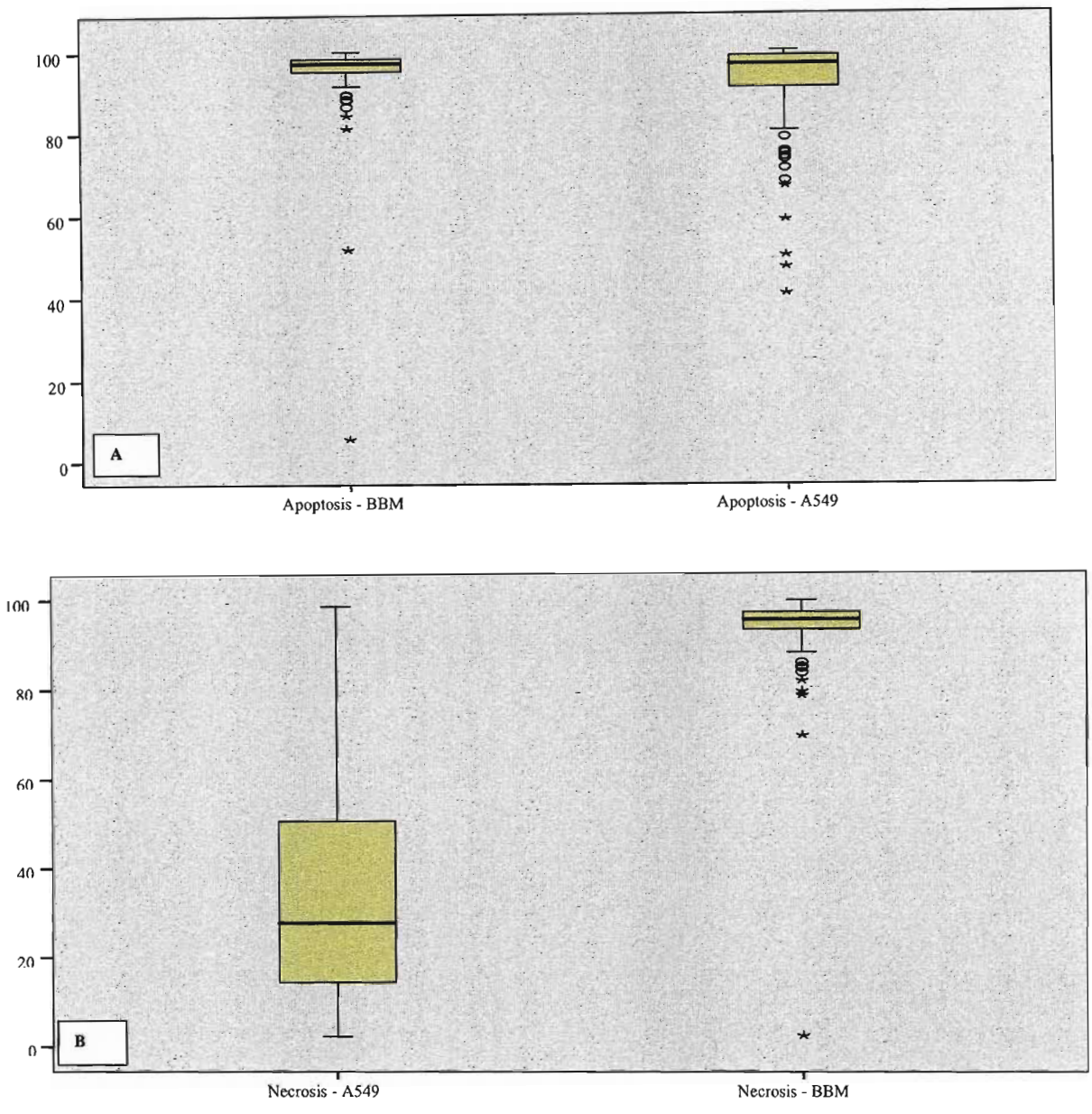

Figure 9: Box plot showing percentage summary of (A) apoptotic and (B) necrotic cells (A549 and BBM). The horizontal line in each box is the median value; thus the bottom of the box is 25th percentile, respectively; the line extended above the box marks 1.5 median values. Circle, values, asterisk, values greater than 1.5 times the media value.

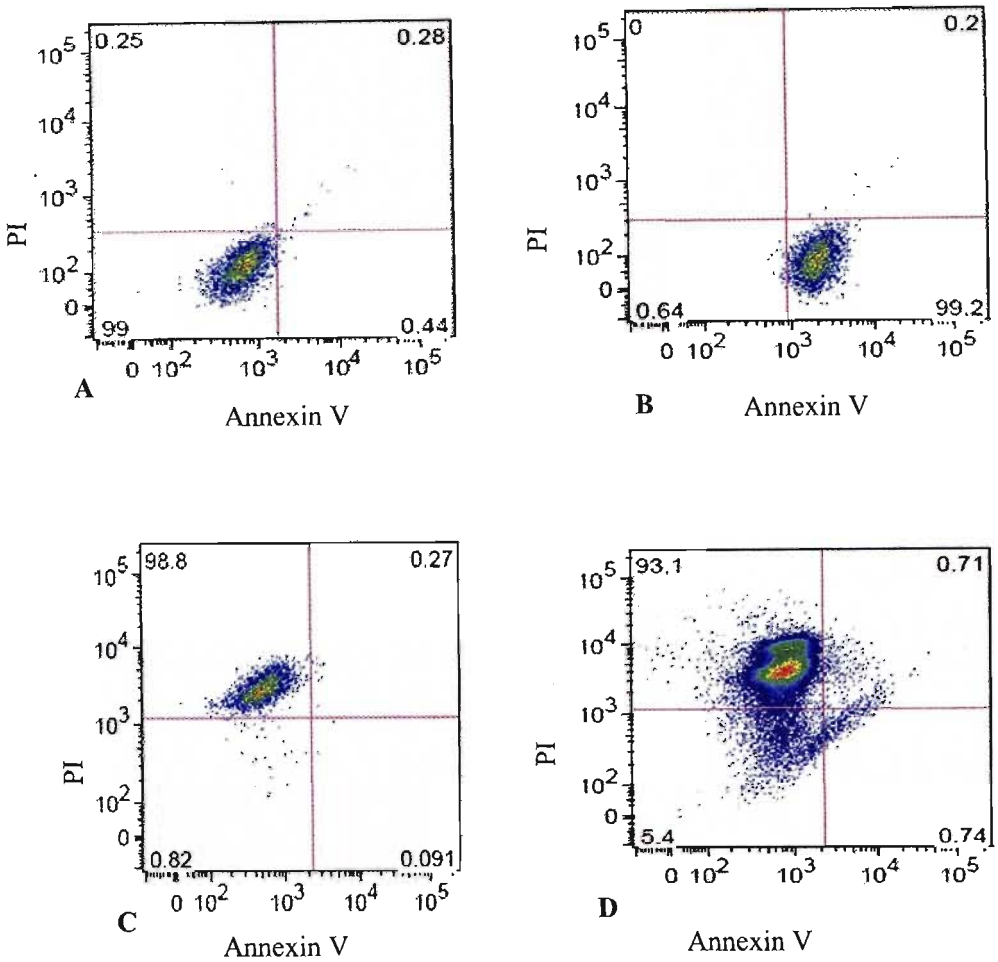

Figure 10: Flow cytometric analyses of A549 controls stained with Annexin-V-FLUOS and propidium iodide. Flow cytometric analysis clearly differentiates dead cells (top right quadrant) with negative for annexin and positive for PI, viable cells (bottom left quadrant) which is negative for both annexin and PI, necrotic cells (top right quadrant) which is double positive for annexin and PI, and finally apoptotic cells (bottom right quadrant) with positive for annexin and negative for PI. A: unstained cells, B: cells stained with annexin-V, C: cells stained with PI and D: actinomycin D (positive control) stained with both (annexin and PI).

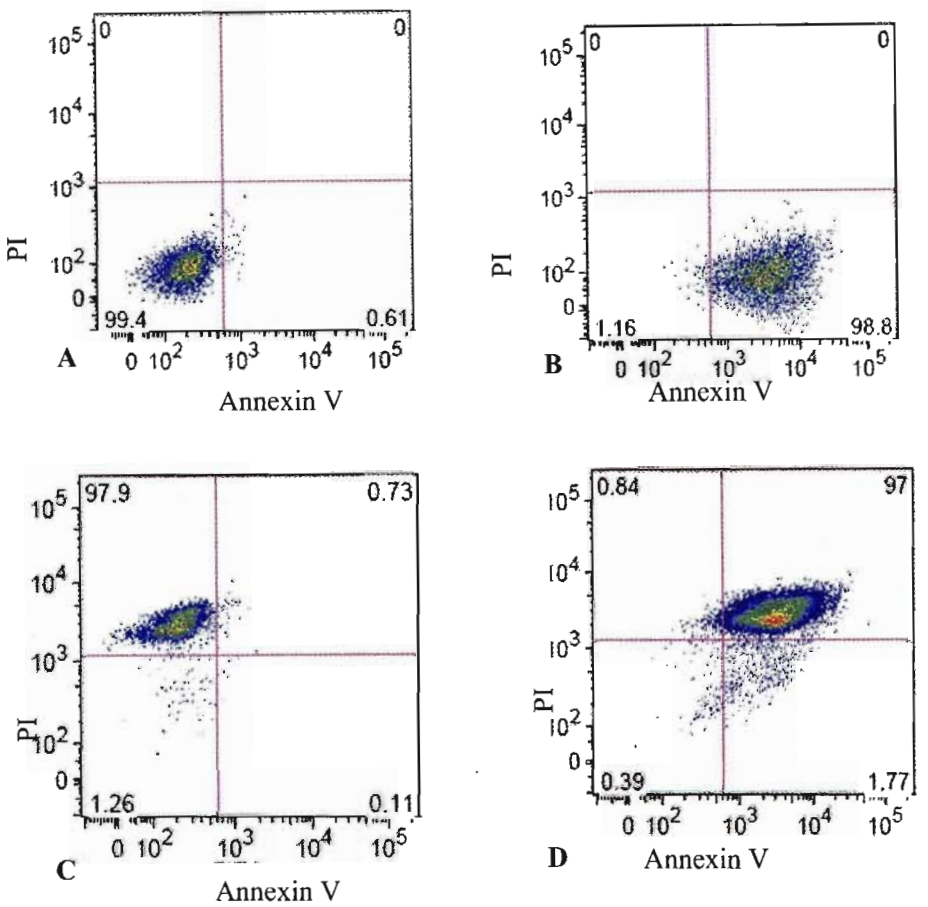

Figure 11: Flow cytometric analysis of BBM controls stained with Annexin-V-FLUOS and propidium iodide. A: unstained cells, B: cells stained with Annexin-V, C: cells stained with PI and D: Cycloheximed (positive control) stained with both (Annexin and PI).

Human cell line apoptosis and necrosis were analysed by flow cytometry and results are given in Figure 12 for A549. It was observed that 25% of cells were necrotic, 62% apoptotic and 13% was non-viable cells. In figure 13 give us BBM results with 99% cells necrotic and only 1% was non-viable. This confirms what has been observed in MTT assay that house dust extract are toxic to cell lines, of which 87% (A549) and 99%(BBM) were in necrotic stage

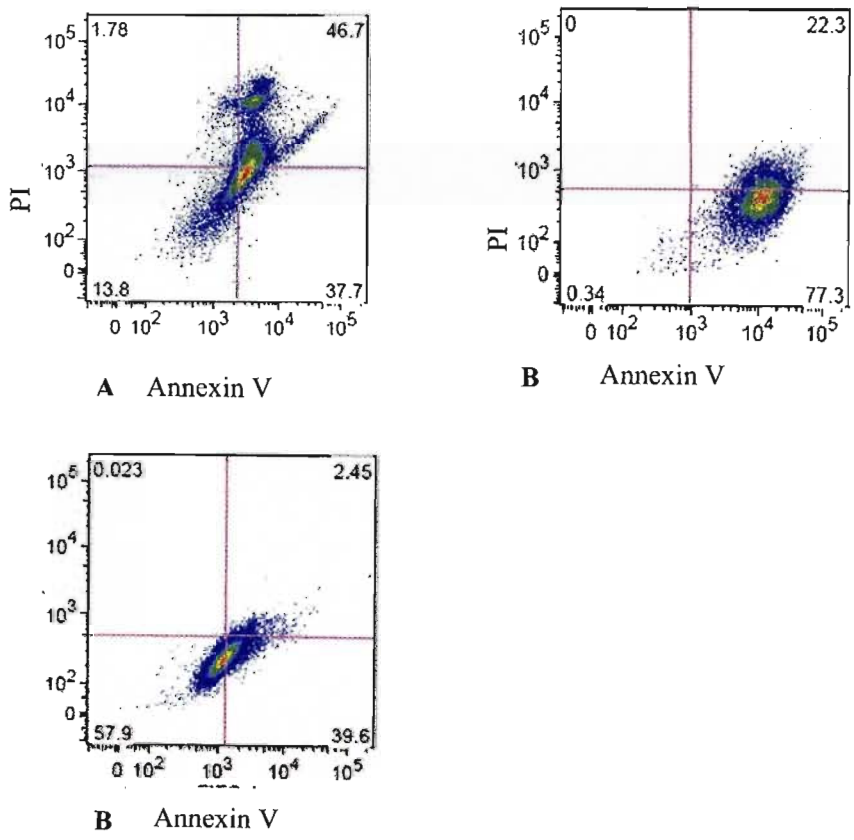

Figure 12: Flow cytometric analysis of A549 treated with dust extract and stained with Annexin-V-FLUOS and propidium iodide. A: 25% necrosis cells, B: 62% apoptosis cells and C: 13% non-viable cells.

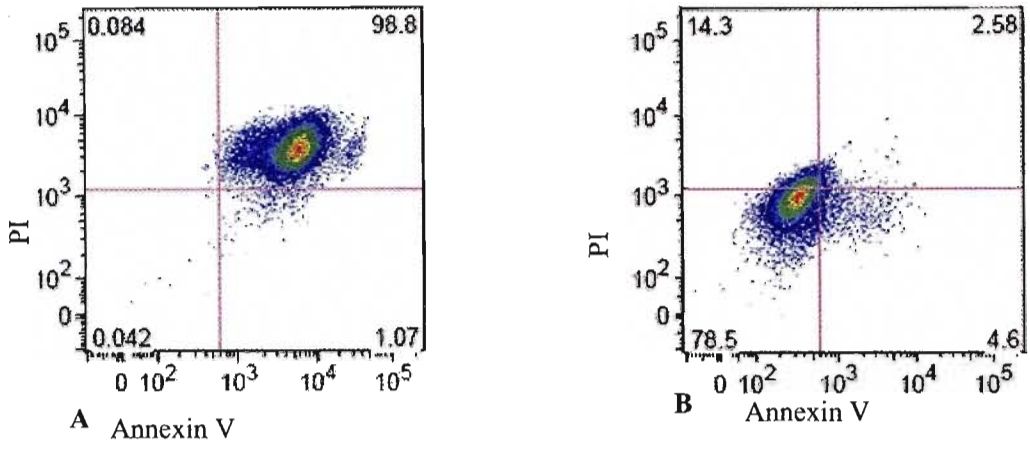

Figure 13: Flow cytometric analysis of BBM treated with dust extract and stained with Annexin-V-FLUOS and propidium iodide. A: 99% necrosis cells and B: 1% non-viable cells.

The cellular apoptotic and necrotic effect was confirmed after cells were exposure to house dust extracts and measured using a fluorescence microscope. The image analysis was done at the University of KwaZulu Natal (UKZN), Optics and Imaging Unit and at the Department of microscope; and the results presented in Figure 14. Cells appear with double colours, the inner colour is orange while the outer colour is green. This is due to the double staining used, namely green (apoptosis) and orange (necrosis). The selected samples that are showed in figure 14 are a representative of cellular changes that A549 cells underwent (treated and controls) during fluorescence microscope observation. Thus, we can say that cell underwent apoptosis cell death stage and some necrosis stage. The limitation for BBM cell, of which is not shown in this diagram due to the lack of cells to attached into cover slips. For this reason we could not report whether or not BBM underwent any cellular changes during treatment. Despite the BBM results not being reported in this experiment, the toxicity results confirm that the house dust extract induced cell death.

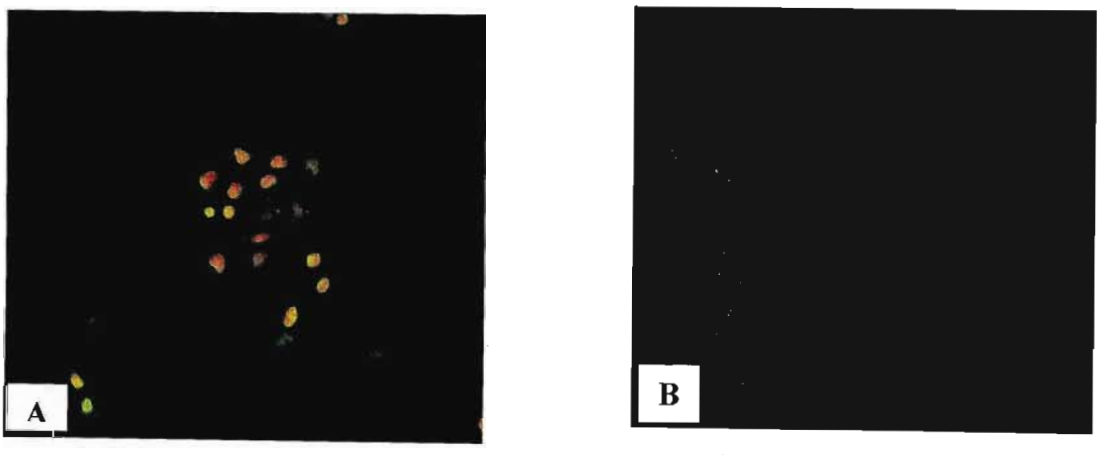

Figure 14: Fluorescent microscopic analysis of apoptosis A549 stained with Annexin-V-FLUOS (green) and propidium iodide (orange). Panel A (A549 sample) and panel B (A549 control).

3.3.3 Multiplex cytokine analysis

3.3.3.1 Method optimization

Human cytokines (IL-4, IL-6, IL-8, IL-13 and TNF-α) were analysed after human cell lines were exposed (1:10 dilution) in house dust extract and measured with Luminex®100 plate reader. The standard curve was calculated using a 4 or 5 parameter logistic fit curves Figure 15 (A549) and Figure 16 (BBM) and which was generated for each cell line. In all standard curves the R-square was between 0.99357 and 0.99971 for all five cytokines analysed, and this is a good correlation. The acceptable criterion for accuracy was set to 75% (lower limit) and 125% (upper limit) represented percentage recovered. In A549 and BBM was observed that the following cytokines (IL-4, IL-6, IL-8, and TNF-α) were reported within acceptable criteria. However, IL-13 was reported to be out of the acceptable criteria for low and upper limits (Tables 10 and 11). This was observed in all concentrations for both parameters in A549 and BBM; the upper limits being (137%; 125.1%) and lower limits (71.5%, 69.9%), respectively. The results indicate the accuracy of Luminex®100 instrument when used to analyse cytokines, and this was observed in all concentrations ranging from high to low for both cell lines.

The quality control was also included in the assay to verify the accuracy of the instrument using the mean fluorescence intensity (MFI) (standards) *versus* pg/ml (samples) and coefficient variance (CV %) was calculated. It must be noted that CV% below 20% is an indication of the reproducibility of the instruments which was achieved for most cytokines for MFI, apart from IL-4 for E and F standards, which were slightly above 20%, which was 24%, and 42%, respectively. Similar results

were observed in MFI for standard curve gradient, IL-4 for E and F standards that were just above 20%, which was 28% and 55%.

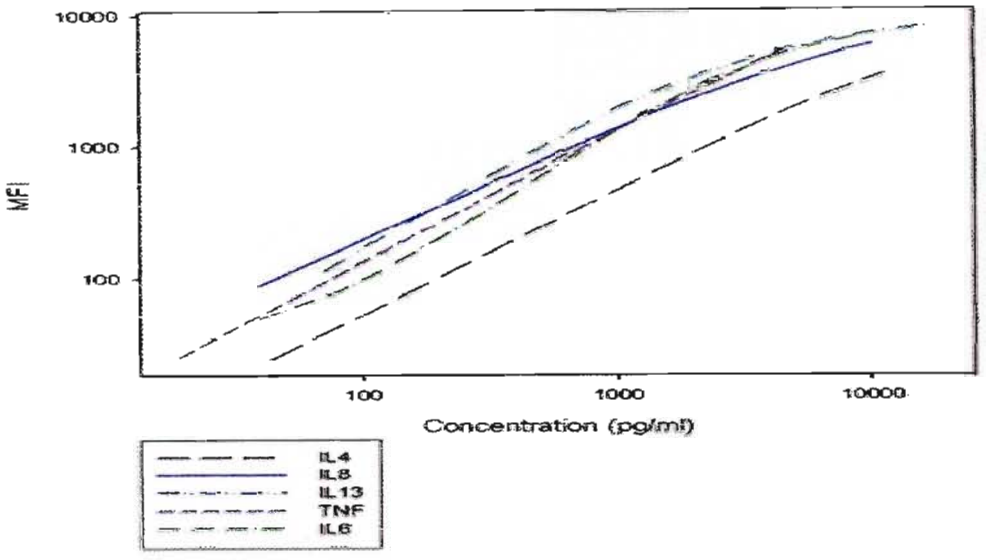

Figure 15: Standard curve plot of A549 (n= 25) samples analysed for (n= 7) concentration versus MFI and regression co- efficient was plotted

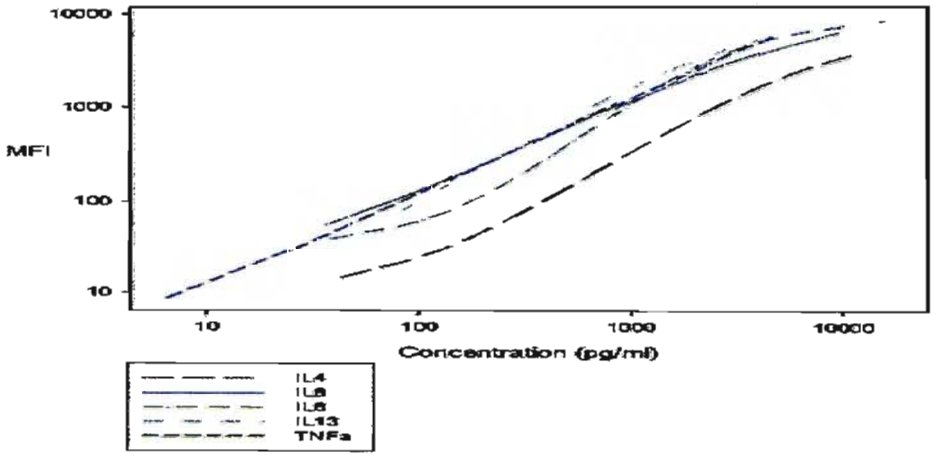

Figure 16: Standard curve plot of BBM (n=25) samples analysed for (n= 7) concentration versus MFI and regression co- efficient was plotted.

Table 8: The percentages recovered for know standards (n=7) that were analysed for A549 cell line.

STANDARDS	IL-4 (%)	IL-6 (%)	IL-8 (%)	IL-13 (%)	TNF-α (%)
A	102	104.7	102.7	*71.5*	1001.1
B	98.3	97.1	96.6	*137*	98.7
C	99.7	106.8	100.6	97.6	103.3
D	102	91	102.3	101.6	96.4
E	99.4	101.6	96.8	96.5	102.6
F	98.9	106.9	102.2	103.7	99.3
G	100.8	96.8	98.3	98.1	100.2

<Lower limit (75)>; <upper limit (125)>

Table 9: The percentages recovered for know standards (n=7) that were analysed for BBM cell line.

Standards	IL-4 (%)	IL-6 (%)	IL-8 (%)	IL-13 (%)	TNF-α (%)
A	99.2	81.3	99.2	*69.9*	96.3
B	101	121.5	99.5	125.1	108.9
C	99.7	107.7	102.9	98.3	96.9
D	102.8	90.9	100.8	106	96.6
E	90.9	92.3	94.3	98.3	99.6
F	124.8	113	105.3	100.6	107.6
G	81.1	94.5	96.5	99	94.6

<Lower limit (75)> ;< upper limit (125)>

3.3.3.2 Cytokines

The analysed house dust extract were grouped according to the dynamic concentration range which was measured in pg/ml for human cell lines and the findings are presented in Table 12. In A549 cell line up-regulated the release of IL-6 and IL-8 and under regulated the other cytokines (IL-4, IL-13 and TNF-α). BBM cell line responded differently to house dust with all five cytokines concerning regulation. It was noticeable that IL-4, IL-8 and IL-13 were within limit of detection. House dust extracts was noticeable to stimulate cytokines released in BBM, while A549 could not release all tested cytokines. The failure of house dust extract to stimulate most cytokines shows that dust may not elicit any allergic reactions.

Table 10: The summary of dynamic concentration (pg/ml) of 5-plex for A549 and BBM cells.

CYTOKINES	DYNAMIC CONCENTRATION RANGE (pg/ml)	
	A549	BBM
IL-4	ND	7.2 – 22.7
IL-6	4.8 - 89.1	7.9 – 10.2
IL-8	116.0 - 14000.0	5.4 – 35.2
IL-13	ND	16.8 - 30.2
TNF-α	ND	3.6 - 4.5

ND, not detected

3.4 Summary

The purpose of this section was designed to answer the questions that arose from the objectives. The occurrence of moulds in residential homes was dominated by *Penicillium spp* and *Rhizopus ssp* with CFU/g below suggested guidelines. The findings do not warrant that buildings are safe for indoor dwellers, but act as indicator for moulds presence indoors. Some of the heavy metals that have been reported in this study were higher or marginally higher than international norms and guidelines and may be of health concern. The toxicity of house dust extract in cells was observed with cell viability below 50% in all five dilutions used in MTT bioassay. Furthermore, cellular DNA damages were also confirmed in the necrotic stage with 99% of BBM cells and 62% for A549 cells. In almost all instance it was observed that house dust extracts hinder the release of cytokines, and this could be due to variety of factors need to be explored.

CHAPTER FOUR -DISCUSSION

4.1 Mould

According to our knowledge, this is the first study in South Africa that describes mould occurrence in settled dust, found in households. The reported top ten predominant genera that were isolated from indoor buildings were among the most commonly isolated in homes: - *Penicillium* ssp, *Aspergillus* ssp and *Rhizopus* ssp. These mould species have been previously reported in suspended and settled house dust in South Africa, Canada and German in problematic and non problematic households (Dales *et al.*, 1997, Sekhotha, 2001, Gansan, 2004; Jafta, 2007).

One South African study reported the occurrence of *Penicillium* spp and *Aspergillus* spp that dominated residential homes in south Durban from suspended dust (Sekhotha, 2001). Gansan, (2004) also reported mould infestation which is related with households dampness which were dominated by the following species *Cladosporium* spp and *Penicillium* spp. The other study was conducted by Jafta (2007) with no particular interest whether homes were problematic or not, and also reported on the *Cladosporium* spp, *Aspergillus* spp, and *Fusarium* spp being dominant in south Durban homes. In Canada Dales *et al.*, (1997) reported that *Aspergillus ssp* and *Penicillium ssp* were dominant in residence with water damaged buildings. Furthermore, similar findings were observed in a German study where *Cladosporium ssp* and *Aspergillus ssp* predominated in settled dust found in households (Jacob *et al.*, 2002).

This study reported considerable high moulds counts in CFU/g when compared with Hicks *et al.*, (2005) in settled house dust. It was noticeable that *Aspergillus* ssp and *Penicillium spp* were classified into the high risk group according to Doe (2003). Despite these moulds counts being reported as "high risk category" the colony counts were below the "contamination guidelines" of 1,000, 000 CFU/g (Brief and Bernath, 1988). The results in this study show that these genera were commonly detected indoors with elevated CFU (Jaffal, 1997, Jacob *et al.*, 2002, Piecková

and Wilkins, 2004; Haas *et al.*, 2007). The reported CFU which are below the suggested guidelines may not pose any risk to an individual, but serves as an indicator of mould presence indoors.

Moulds infestation in residential homes pose health risk such as allergic asthma and toxins production with know genera that were also identified in this study (predominant). Samson *et al.*, (1994) and Horner *et al.*, (2004) reported on *Absidia ssp, Chaetomium spp, Curvularia spp, Helminthosporium ssp, Rhizopus ssp,* and *Penicillum ssp* with the ability to cause allergic reaction in humans. Horak *et al.*, (1996), Verhoeff *el al.*, (1997) and Pieckova and Jesenskà (1999) reported on *Alternaria* ssp, and *Cladosporium* ssp cause extrinsic asthma while *Aspergillus* ssp can produce ochratoxins. In this study *Fusarium* spp was reported and widely distributed in soil and can infect food plants. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes; and the health effects on human can range from opportunistic infections. Other species of *Fusarium* reportedly cause disseminated infection which is aggressive in particular to the immune compromised individuals and penetrate the entire body and bloodstream (Nelson *et al.*, 1993, Nelson *et al.*, 1994; Placinta *et al..*, 1999).

In this study the isolation and identification of outdoor mould was not done. When studying indoor moulds infestation it's crucial to understand the influence of outdoor moulds. However, *Cladosporium* ssp and *Alternaria* spp were isolated in this study which was reported by Jacob *et al.*, (2002) to dominate outdoor environment. These moulds isolated both from indoor and outdoor environment (Samson *et al.*, 1994, Hameed *et al.*, 2004; Horner *et al.*, 2004). Therefore, this indicates mobility of spores from outdoor into the indoor environment. The presence of moulds indoors is of health concern as the individuals exposed to them many develop negative health effects. The mould infestation in this study was widely distributed in all households sampled.

4.2 Heavy metals

Heavy metals were present in varying quantities and ranges in the households in this study. Generally, variations of the mean levels among communities' studies were within a small range. The exception was noticed with lead in Newlands West where the levels of this metal were substantially higher than the mean levels for other communities in the north. Generally, Pb levels was within suggested guidelines (500µg/g) but was exceeded in some households in Merebank and Newlands West. The Pb results in this study were relatively low when compared with these observed in literature. For example, one of the South African study reported highest mean of 3940 µg/g, and samples were taken from wall peeled paints (Mathee *et al.*, 1993). The Korea study reported Pb mean of 2350.0 µg/g in house dust environment near subway stations (Kim *et al.*, 1998). The dispersion of Pb indoors has been reported to cause chronic illnesses such as neurological impairment to humans (Manser *et al.*, 1989, Shen *et al.*, 2001; Creel, 2002). In south Durban, industries have been pin-pointed as the source of contamination (Robins *et al.*, 2002; Hallow, 2003).

The As mean levels in this study were generally within suggested residential soil guidelines 20 µg/g. Arsenic was comparable with one study in Germany (mean =2.6 µg/g) on the house dust taken near the smelting industry (Meyer *et al.*, 1999). Whilst in Slovakia, were marginally high As levels reported (mean =18.3 µg/g) in house dust (Keegan *et al.*, 2002). Arsenic has been associated with the causes of chronic illnesses such as cancers namely; colon, liver and bladder (Ng *et al.*, 2003).

Mercury levels were within the guideline of 6.6 µg/g, thought some of the samples were notably exceeded by households in Newlands West (north Durban community). It can also be noted that Hg levels was substantially higher in this study than reported in previous studies. In Northeast

China reported fairly lower Hg (mean=0.097 μg/g) in the urban residential (Li *et al.*, 2006). Bastos *et al.*, (2004) reported Hg levels (mean= 3.22 μg/g) in a contaminated gold shop, which are marginally lower than current study. Despite the fact that mercury levels were generally lower than the levels on reported guidelines, but its occurrence indoors is a health concerns for the indoor dwellers with reported acute toxicity (Chapman and Chan, 2000).

In this study, it was observed that Pb and As were significant for south and north communities. Mercury was marginally higher in the north, but statistically insignificant. Heavy metal was found in moderate to high levels in households in the Durban communities. Several households consistently exceeded suggested guidelines for various pollutants. The respective communities in Merebank, Newlands West and Bluff need particular attention and further investigation. The important difference was noticed in south communities near the industrial areas with respect to Pb and As. The results in this study may suggest that industrial pollution may be a contributory factor in the elevated levels of heavy metals in households and further studies should be undertaken, particularly in Merebank area to further confirm findings.

This study successfully measured the proposed problematic heavy metals indoors. However, the scope of this study did not extend to measurement heavy metal personal exposure. Future research pertaining to the occurrence of heavy metals indoors should measure heavy metals in humans to assess the risk of causing negative health effects. The findings may contribute towards the development of South African guidelines.

4.3 Cytotoxicity of household dust extract

4.3.1 Apoptosis and necrosis measurement

The degree of cell activation membrane lysis and mitochondrial ultra-structural toxicity was observed when cells were treated with various house dust extract dilutions. The ability of house

dust extract to lower cell viability was observed in this study with cell reduction less than 50% following house dust extract exposure. The apoptotic and necrotic ability of cells when treated with house dust extract was further verified by Annexin-V-Fluos assay as to confirm what has been reported in MTT assay. Apoptosis is the key feature of programme cell death which leads to variety of cellular changes such as blebbing, changes in cell membrane and necrosis can be defined as the traumatic cell death that can results in acute cellular injury and noticeable by the destruction of cellular DNA (Oshimi et al., 1996).

In this study it was observed that human bronchial epithelia cell lines underwent a degree of apoptosis and necrosis. A549 cells responded differently towards the house dust extract with 62% apoptosis; whilst, 99% of BBM cells underwent apoptosis. The cellular changes were further confirmed (fluorescent microscope) for A549 cells, of which was not seen in BBM cells due to the difficulties of cell attachment on cover slips after staining. Ishii *et al.*, (2004) and Chen *et al.*, (2008) reported on the cellular death characteristic such as poor cellular shrinkage, and round poorly adherent cells; unattached round shape cells was evident in this study. The degree of cell death was different between the two cell lines. This does make sense due to fact that one is normal (BBM) and A549 is cancerous cell line. Generally, objectives of this study were met and we can safely say that house dust showed toxicity to cells.

4.3.2 Multiplex cytokines

This study showed that house dust extract is toxic to human cell lines, and we thought it is necessary to understand in what extent the inflammatory potential can be triggered. The cytokines serve an important role in the non-specific defence external against insults

It was observed in this study that A549 cells up-regulated the release of pro-inflammation (IL-6 and IL-8) cytokines. In one study, IL-8 production depended on TNF-α stimulation (Lee *et al.*,

2005), and the similar effect was noticed by the increase production of IL-6 and IL-8 (Zhang *et al.*, 2005). However, Saraf *et al.*, (1999) found that IL-6 and IL-8 are dose dependent and receptors play a critical role in the antigen attachment and expression. In this study, TNF-α was not expressed and cytokines released was through house dust extract stimulation. These cytokines are pro-inflammatory cytokines with important differences in their production and receptors (Xing *et al.*, 1998). IL-6 produced by T-cells and macrophages with ligand-binding receptor (Heinrich *et al.*, 2003); and IL-8 produced by epithelial cells, endothelial cells and macrophages with chemokines receptors (Köhidai and Csaba, 1998, Wolff *et al.*, 1998; IUIS, 2002). This explains considerable differences that was reported in A549 regarding these cytokines.

In this study it can be seen that BBM cells express all cytokines at low concentrations. Despite this further research is required as to understand its mode of action. Previous studies reported lower or undetectable levels of cytokines using normal cells with noticeable increase in cytokines production after stimulation, and similar effects were noticed with A549 (Fuji *et al.*, 2001, Veranth *et al.*, 2004; Lee *et al.*, 2005). The differences observed in the study regarding expression or suppression of cytokines by cells is not clear. It can be postulated that the variation observed could be linked to number of factors such as functionality difference or ability of test substance (house dust extract in this case) to attenuate or suppress cells to respond towards foreign materials being exposed too. In contrast to this, BBM released IL-4 and IL-13 which share functional subunits for signal transduction (Zurawski *et al.*, 1993; Doucet *et al.*, 1998). This cytokines act as anti-inflammatory properties and are released in airway disease (Murata *et al.*, 1998; Wynn, 2003).

The expressions of cytokines that are involved in allergic inflammation were observed to be reduced in this study. Human cell lines did not show any allergic reaction, further studies should be conducted as to understand air pollution disease such as allergic.

CHAPTER FIVE -

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The main area of interest in this study was to conduct toxicological analysis of house dust extract collected from inside selected Durban residential buildings. The scope of this study was to measure indoor pollutants such as moulds allergens, heavy metals and toxicological effects of human bronchial epithelial cell lines.

In this study we identified and quantified indoor moulds. The outdoor mould investigation was not conducted. This would have enabled a better understanding of the relationships between these two environments. The indoor moulds that were predominating in this study were identified and also reported on the ones that are usually found outdoors. This suggests that outdoor moulds migrates indoors and may negatively impact this environment.

The occurrence of heavy metals in house dust was measured and compared with suggested guidelines by regulatory bodies. The results were discussed in line with findings in literature. Three communities in the study undertaken in the Durban region consistently exceeded suggested guidelines for various pollutants. These communities, two in the south and one in the north, need particular attention with respect to the source of heavy metals.

Human cell lines shown to be toxicity towards house dust extract. Furthermore, it was observed that A549 cells up-regulated the release of some cytokines and BBM down regulated the release of other cytokines, and most cytokines were reported in low concentrations. Therefore, we cannot say that house dust extract responsible in the production of primary line of defence mechanism. The modes in which the cells are stimulated by house dust extract to release cytokines need further investigation. It is often true that the house dust extract used in the

laboratory is higher than what humans can take in a given time. Through this, it can be postulated that human exposure to contaminated indoor air pollution may be detrimental to humans and that the duration of exposure is critical. The study has shown us that house dust extract is toxic to cells and humans in the households in this study are at risk from this house dust.

5.2 Recommendations

It is recommended that households should be free from solvents that emit heavy metals and it is necessary to eliminate moisture environment which becomes a good environment for mould growth. In the latter example, it is recommended that cleaning strategies be implemented, such as vacuuming the households as to minimize suspended particles being introduced indoors.

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APPENDICES

APPENDIX A: Dust sampling protocol

(Developed & supplied by Robins T, Baldwin G & CAAA project group – Michigan, USA)

PURPOSE: Dust sampling provides information about possible sources of biological and chemical agents in buildings as well as the composition and relative concentration. Dust, house dust, or settled dust are terms used to describe the material that collects on horizontal surfaces in textiles, upholstered furniture, carpets and other surfaces. Allergens, fungal spores, metals, and other compounds can be determined from the collected dust.

FIELD EQUIPMENT/ SUPPLIES:

1. Vacuum cleaner or suction device
2. Sampling nozzle (and filter papers, gloves & breathing masks)
3. Twizers for handling filter paper (8 μm retention).
4. Ziplock bags and aluminium foil
4. Cotton gause pad, e.g.10 cm x 10cm.
5. Rubbing alcohol, 70% ethanol or isopropanol.
6. Refrigerant packs: NOTE: Keep samples cool, but protect from freezing.
7. Label tags, marker & string (1 m^2)

After the walk-through, you will learn which areas area suitable for dust sampling. Specific things will be sampled: (1) the bedding [including their pillow(s) & mattress] (2) floor area nears the bed and (3) soft furnisher (such as fabric covered couch or chairs).

SAMPLING INSTRUCTIONS

In sleep area find an area on the floor near the sleeping location that is a representative area. *Areas to avoid include a pet's bed and areas near the windows, UNLESS the person's are directly exposed to it when sleeping (for example, the person's head is located right by the window).*

In the living room identify furniture that accumulates dust especially horizontal surfaces such as the clothed furnishers, desks, tables, window seals etc. should be sampled.

With the string, form a square around the area you selected and tape down the corners. *If the area is not big enough to form a square, try a rectangle, but keep it as close to a four-sided shape as possible!*

To sample the bedding, you may have to straighten the bedding a little. You will be sampling the pillow(s) as well as the piece of bedding that is on top--this may be, for example, their comforter, blanket, or flat sheet. If you are unsure, ask the participant (parent or caregiver). If there is more than one pillow, you can sample them if you have time; otherwise, focus on the top one.

Plug in the vacuum cleaner, and make sure the cord will reach the marked area and the bed. Use the extension wand if necessary. If you need to unplug something in order to plug in the vacuum cleaner, try not to unplug electric clocks, or any other appliances that are in use.

Clean the end of the vacuum hose with an alcohol wipe. Also clean the entire nozzle attachment with the alcohol wipe to ensure that there is no contamination from previous samples. Please

clean the attachment well, since you will be sampling from the child's bedding.

Select a filter for dust collection. From the sheet of ID labels remove a label and place it on the white sticker on the bag, by NAME. Place another ID label on the Dust Log Sheet.

Place the filter into the nozzle. Be sure the entire filter is aligned the right way in the nozzle.

Attach the nozzle to the hose, and make sure that the collector is tight and secure in between the nozzle and hose. If the filter is not correctly aligned, some of the dust sample may get sucked into the vacuum cleaner!

Note: Wear dust mask before starting to collect dust

Turn the vacuum on. Remember to record the start time on the Dust Log Sheet.

First slowly run the nozzle up and down the pillow. Repeat on the other side. Do each side twice.

Vacuum the top piece of bedding that you have straightened. The area vacuumed on the bedding can be the same size (or close to it) as the area marked off by the string. Vacuum for 3 minutes.

Next, vacuum the square area on the floor that you marked off with the string. Make sure the nozzle is in full contact with the floor. Move the nozzle over the sample area slowly. Vacuum for 2 minutes.

After you have finished, hold the hose upright with the nozzle in the air--and turn off the vacuum.

Gently remove the nozzle, and make sure that no dust falls out of the collector.

Gently remove the filter collector from inside the nozzle.

Fold the filter over, place it on an aluminium foil and fold it in. Place the filter into the zip lock bag.

Be sure that the Dust Log Sheet has the ID label, temperature and humidity, your name, the date and time of collection, and any notes that are important. After it is completed, fold the sheet and place it inside the other ziplock bag. Place this ziplock bag in bag with the collected dust.

A zip lock bag for trash has to be provided. Please use this bag for waste from tape, alcohol swab wipes, etc.

Additional Notes:

Please try to avoid vacuuming any wet or damp areas on the bed or rug.

If the floor in the child's sleeping area is not carpeted, but there is a small rug by where they sleep, sample the rug. Please note this on the Dust Log Sheet.

Remember to clean the hose and the nozzle well before each use.

If a collector accidentally gets vacuumed into the vacuum cleaner in the middle of your sampling, replace with a new collector and continue the sampling. Retrieve the other collector

from the vacuum bag after you have finished. Both collectors can be put into the same bag, but seal each collector with their own sticker. Indicate this on the Dust Log Sheet.

Things to note on the Dust Log Sheet:

If you forgot to wash your hands after handling pets

If the participants are uncomfortable with you sampling the bedding, or if they vacuumed the bedding for you

If you sampled a rug by the sleeping area (as mentioned in item #2 in "Additional Notes")

If a collector accidentally gets sucked into the vacuum

If you lose some of the dust sample

Anything else that you would be viable to bring attention to

DUST LOG SHEET

ID Label: _____

Physical Address: _____

Temperature: _____

Humidity: _____

Interviewer's name: _____

Date of collection: _____

Starting time of collection: _____

Notes:

APPENDIX B: Mould confirmation results

Predominate isolates that was sent at Allerton Provincial Laboratory for the confirmation and results are given here below:-

SAMPLE REF NO	IDENTIFIED	CONFIRMED
142323100	*Aspergillus ochraceus*	✓
141019100	contaminated	×
241034100	*Fuserium culmorum*	✓
343540100	*Penicillium variable*	✓
342532100	*Penicillium spinulosum*	✓
441420100	*Aspergillus ochraceus*	✓
541140100	*Aspergillus niger*	✓
541149100	*Penicillium spinulosum*	✓
541122100	*Alternaria spp*	✓
642415100	*Fuserium culmorum*	✓
641178100	contaminated	×
6421182100	*Alternaria alternate*	✓
733565100 (a)	*Penicillium fellutnum*	✓
733567100	contaminated	×
741215100 (b)	*Fuserium solani*	✓

APPENDIX C: Mercury analysis with method 43.

UMGENI	Methods Manual Laboratory Services	Date of Issue: 14/08/2006	Ed/Rev.:02/02
MERCURY			Method 34

Reviewed by:_____ Technical Supervisor/Section Head

Approved by:_____ Technical Manager, Laboratory Services

INTRODUCTION

Organic and inorganic mercury salts are very toxic and their presence in the environment, especially in water, should be monitored.

All forms of mercury, including organic derivatives, are oxidised to ionic mercury by acidic potassium permanganate. Inorganic mercury is rapidly reduced by sodium borohydride in an acidic solution to mercury vapour. The vapour is purged continuously with argon into an unheated quartz tube in the light path of an atomic absorption spectrometer where absorbance is determined at 253.7nm.

SCOPE

This method is applicable for the analysis of trace quantities of mercury in clean waters and wastewaters.

INTERFERENCE

Water vapour can result in loss of sensitivity and baseline drift. Condensation of water can occur in the measuring cell and this can be prevented by using a drying tube on the inlet to the cell.

SAFETY, HEALTH AND ENVIRONMENT

Nitric acid, hydrochloric acid and sulphuric acid

Potassium dichromate

Potassium permanganate

Mercury

Sodium borohydride.

See the manufacturer's handbook for instrument related hazards.

Ensure that you are familiar with the dangers and treatment associated with each of the above substances. (Methods Manual and SOP 19).

The preserved samples and waste are toxic and must be disposed of as per SOP 19a.

SAMPLE COLLECTION AND PRESERVATION

Samples should be collected in 250 mL glass bottles with 1mL dichromate solution (refer 7.7) and 1mL nitric acid (refer 7.6) per 100 mL sample.

The preserved samples and waste are toxic and must be disposed of as per SOP 19a.

APPARATUS

Atomic Absorption Spectrometer with a 253.7 nm mercury lamp.

Hydride Generator

Vapour generation Accessory with pump system.

Sampler

Autosampler or manual sampling.

6.4 Glassware

Certified measuring cylinders, volumetric glassware and beakers, as required.

Pump Tube Sizes:

Sample line	:purple-white	(3 pump tubes)
Acid line (5M HCL)	:orange-orange	
Borohydride line	:orange-orange	

7. REAGENTS

8. PREPARATION OF STANDARDS

9. PREPARATION OF ANALYTICAL QUALITY CONTROL (AQC)

10. ANALYTICAL PROCEDURE

11. CALCULATION OF RESULTS

The instrument is calibrated by means of standards from which a calibration graph is established. Sample concentrations are read from the calibration graph. The instrument does not perform automatic dilutions. Calculations for manual dilutions need to be indicated on the run-sheet.

SOURCES OF ERROR

Contaminated glassware.

Water vapour.

Incorrect pump tubes.

Old $NaBH_4$ salt.

REFERENCES

SCA Method: Mercury in waters, effluents and sludge's by Flameless Atomic Absorption Spectrophotometer (1978 Version).

Refer to the instrument operating manuals for further details regarding the setting up and running of the AA.

APPENDIX D: Lead analysis with method 102

UMGENI	Methods Manual Laboratory Services	Date of Issue: 07/01/2005	Ed/Rev.:02/02
NICKEL, LEAD, CADMIUM, CHROMIUM, SILVER, COBALT, BERYLLIUM, VANADIUM, MOLYBDENUM, ALUMINIUM			Method 102

Reviewed by:_____ Technical Supervisor/Section Head

Approved by:_____ Technical Manager, Laboratory Services

1. INTRODUCTION

The effects of metals in water range from beneficial and troublesome, through to dangerously toxic. Metals may be determined satisfactorily by atomic absorption spectroscopy, inductively coupled plasma spectroscopy (ICP) or colorimetric methods. Inductively coupled plasma techniques are applicable over a broad linear range and are especially sensitive for refractory elements.

In this procedure, nitric acid extractable nickel (Ni), lead (Pb), cadmium (Cd), chromium (Cr), silver (Ag), cobalt (Co), beryllium (Be), vanadium (V), molybdenum (Mo) and aluminium (Al), and are determined by measuring the emission signal using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

2. SCOPE

This method is suitable for the analysis of the above-mentioned elements in clean waters.

3. INTERFERENCES

Spectral overlap from matrix elements can be problematic in complex matrices. This type of interference can be avoided by selecting an alternate wavelength. Nebulisation interferences and crystallization in the sample injection tube of the torch must be considered when analyzing viscous, high density samples or samples with very high dissolved solids. If these interferences are present, they can be compensated for by sample dilution, by using matrix-matched calibration standards or by applying the standard addition technique.

4. SAFETY, HEALTH AND ENVIRONMENT

Nitric acid.

See the manufacturer's manual for instrument related hazards.

Ensure that you are familiar with the dangers and treatment associated with each of the substances used. (Methods Manual and SOP 19).

Exercise caution when handling wastewater samples which pose serious health risks. It is recommended that gloves be worn. Do not inhale nor ingest. Upon ingestion, proceed to hospital immediately.

5. SAMPLE COLLECTION AND PRESERVATION

Samples should be collected in polyethylene bottles with 2mL nitric acid (refer 7.1) per 100 mL sample.

6. APPARATUS

6.1 Inductively Coupled Plasma Spectrometer.

6.2 Ultrasonic nebuliser.

6.3 Autosampler or manual sampling.

Centrifuge

Glassware: Certified measuring cylinders, A Grade volumetric glassware, test tubes and beakers as required.

7. REAGENTS

8. PREPARATION OF STANDARDS

9. PREPARATION OF AQC

10. ANALYTICAL PROCEDURE

11. CALCULATION OF RESULTS

The instrument is calibrated by means of standards from which a calibration graph is established. Sample concentrations are read from the calibration graph. The instrument does not perform automatic dilutions. Calculations for manual dilutions need to be indicated on the run-sheet.

SOURCES OF ERROR

Problems relating to the ultrasonic nebuliser.

Gas leaks.

Contamination of glassware.

13. REFERENCES

ICP-OES operation/instrument manual

APPENDIX E: Arsenic analysis with method 93

UMGENI	Methods Manual Laboratory Services	Date of Issue: 14/08/2006	Ed/Rev.: 02/03
ARSENIC			Method 93

Reviewed by:_____ Technical Supervisor/Section Head

Approved by:_____ Technical Manager, Laboratory Services

1. INTRODUCTION

Severe poisoning can arise from the ingestion of as little as 100 mg arsenic trioxide; chronic effects may result from the accumulation of arsenic compounds in the body even at low intake levels.

Arsenic is reduced to its hydride by sodium borohydride in an acidic solution. The hydride and excess hydrogen formed are flushed through, from the gas/liquid separator, by a stream of argon into a chemically generated hydrogen flame. The hydrides are atomised and the resulting atoms detected by atomic fluorescence.

2. SCOPE

This method is applicable for the analysis of trace quantities of arsenic in clean waters.

3. INTERFERENCES

Interferences are minimized as the volatile hydride is separated from the solution, leaving behind many potentially interfering substances. In cases of mild interference, the standard additions method will largely correct determined values.

4. SAFETY, HEALTH AND ENVIRONMENT

Hydrochloric acid.

Sodium borohydride.

This method generates a fair amount of hydrogen. It is therefore important to have a good extraction system operating at all times to prevent explosions.

Ensure that you are familiar with the dangers and treatment associated with each of the above substances. (Methods Manual and SOP 19).

5. SAMPLE COLLECTION AND PRESERVATION

Samples should be collected in polyethylene bottles with 1mL hydrochloric acid (refer 7.4) per 100 mL sample.

6. APPARATUS

6.1 Detector

PS Analytical Excalibur Detector.

6.2 Hydride Generator

PS Analytical Automatic Hydride Generator.

6.3 Sampler

PS Analytical autosampler or manual sampling.

6.4 Glassware

Certified measuring cylinders, volumetric glassware and beakers as required.

6.5 Pump Tube Sizes:

Sample line	: orange
Acid line	: orange
Borohydride line	: purple

6.6 Reagent Filtration kit

Buchner flask, funnel and 0.45 µm (47 mm diameter) filters.

7. REAGENTS

9. PREPARATION OF ANALYTICAL QUALITY CONTROL (AQC)

10. ANALYTICAL PROCEDURE

11. CALCULATION OF RESULTS

The instrument is calibrated by means of standards from which a calibration graph is established. Sample concentrations are read from the calibration graph. The instrument does not perform automatic dilutions. Calculations for manual dilutions need to be indicated on the run-sheet.

12. SOURCES OF ERROR

Water vapour that is present with the generated metal hydride in the flame.

Incorrect pump tubes.

Incorrect acid concentration in the blank.

Old $NaBH_4$ salt.

REFERENCES

Manufacture's Operating Manuals SOP 04a

Cell Proliferation Kit I (MTT)

Colorimetric assay (MTT based) for the non-radioactive quantification of cell proliferation and viability

Cat. No. 11 465 007 001
1 Kit (for 2500 tests)

Version Sept. 2005
Store at –15 to –25°C

1. Kit contents

Bottle	Label	Contents
1	MTT labeling reagent	• 5 vials containing 5 ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) labeling reagent (1x). • 5 mg/ml in phosphate buffered saline (PBS). • non-sterile • ready to use.
2	Solubilization solution (1×, ready-to-use)	• 3 bottles with 90 ml • 10% SDS in 0.01 M HCl.

2. Introduction

2.1 Product overview

Assay principle

The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells (fig. 1) (6, 7, 35).

This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH (36). The formazan crystals formed are solubilized and the resulting colored solution is quantified using a scanning multiwell spectrophotometer (ELISA reader).

This ensures a high degree of accuracy, enables on-line computer processing of the data (data collection, calculation and report generation) and, thereby, allows the rapid and convenient handling of a high number of samples.

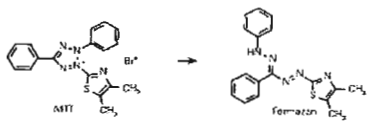

Fig. 1: Metabolization of MTT to a formazan salt by viable cells.

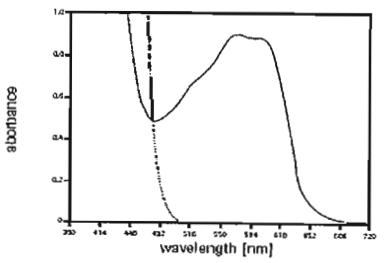

Fig. 2: Comparison of UV-spectra of MTT labeling reagent (dotted line) and the formazan salt after solubilization with solubilization solution.

Basic steps

Stage	Description
1	Cells, grown in a 96 well tissue culture plate, are incubated with the yellow MTT solution for approx. 4 h.
2	After this incubation period, purple formazan salt crystals are formed. These salt crystals are insoluble in aqueous solution, but may be solubilized by adding the solubilization solution and incubating the plates overnight in humidified atmosphere (e.g. 37°C, 6.5% CO_2).
3	The solubilized formazan product is spectrophotometrically quantified using an ELISA reader. An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed, as monitored by the absorbance (see fig. 3).

Background information

The determination of cellular proliferation, viability and activation are key areas in a wide variety of cell biological approaches. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Such an example is based on the capability of the cells to incorporate a radioactively labeled substance ([^{3}H])- thymidine), or to release a radioisotope such as [^{51}Cr] after cell lysis. Alternatively, the incorporation of 5-bromo-2'-deoxyuridine (BrdU)* in place of thymidine is monitored as a parameter for DNA synthesis and cellular proliferation in immuno-histo-and cytochemistry, in a cell ELISA and FACS analysis. (kits and reagents for these applications are available from Roche Applied Science). Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts (e.g. MTT, XTT, WST-1) are especially useful for assaying the quantification of viable cells, because they are cleaved to form a formazan dye (fig. 1; for UV absorbance spectrum, see fig. 2) only by metabolic active cells.

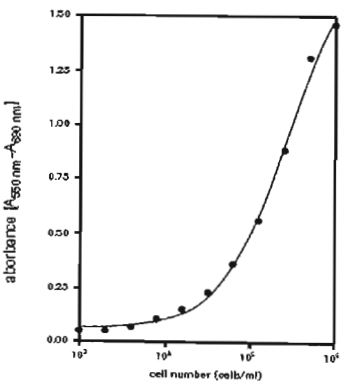

Fig. 3: Effect of different numbers of cells on color formation (example given, using Ag8 cells).

Application

The non-radioactive, colorimetric assay system using MTT was first described by Mosmann, T. et al. (1) and improved in subsequent years by several other investigators (2–6).

The assay is designed for the spectro-photometric quantification of cell growth and viability (1, 3, 5–7) without the use of radioactive isotopes.

- It is used for the measurement of cell proliferation in response to growth factors, cytokines and nutrients (1–3, 6, 8–12) (see fig. 4).
- The MTT assay is also useful for the measurement of cytotoxicity. Examples are the quantification of tumor necrosis factor-a or -b effects (13, 14). (see fig. 5) or macrophage induced cell death (15, 16) and the assessment of cytotoxic (17–34) or growth inhibiting agents such as inhibitory antibodies (see fig. 6).
- For the replacement of the radioactive (^{51}Cr)-release cytotoxicity assay, protocols using MTT have been developed. The MTT assay is as sensitive as the radioactive method, but shows a significantly lower background especially after long term incubation (34).
- The MTT assay can also be used to study cell activation (4).

Storage and stability

Stable at -15 to -25°Cuntil the expiration date printed on the label.

Note: Protect from light. Repeated thaw-freeze cycles do not affect product stability. Precipitates may form during shipment or storage, in which case the container should be warmed to 37°C and thoroughly mixed.

After thawing, the MTT labeling reagents may be stored protected from light at+ 2 to +8°C for up to 4 weeks, in which case a sterile filtration of the reagent is r-ecommended.

Advantages

Compared to radioactive isotope techniques, the Cell Proliferation Kit I (MTT) shows the following benefits.

Benefit	Feature
Safe	No radioactive isotopes are used.
Accurate	The absorbance revealed, strongly correlates to the cell number, (see fig. 3).
Sensitive	Low cell numbers are detected (see fig. 3).
Fast	The use of multiwell-ELISA readers allows for processing a large number of samples.
Easy	No washing steps and no additional reagents are required.

3. Protocols and required material

3.1 Assay procedure

Overview

Please refer to the following table.

Step	Description	Volume/ well	Time/ Temp
	Perform tissue culture using 96 well microplates (tissue culture grade, flat-bottom)	100 µl	24-96 h 37°C.
1	Add MTT labeling reagent and incubate in a humidified atmosphere	10 µl	4 h 37°C.
2	Add solubilization solution and incubate in a humidified atmosphere	100 µl	overnight 37°C
3	Evaluate microplate with the use of an ELISA reader at 550–600 nm with a reference wavelength of >650 nm.		

Handling instruction for larger volumes

If for the initial incubation of the cells a larger volume of culture medium is required, increase the amount of MTT labeling reagent correspondingly (e.g. 20 µl MTT labeling reagent, when cells are cultured in 200 µl culture medium).

Protocol

Please refer to the following table.

Note: If for the initial incubation of the cells a larger volume of culture medium is required, increase then amount of MTT labeling reagent correspondingly (e.g. 20 µl MTT labeling reagent, when cells are cultured in 200 µl culture medium).

Step	Action
1	Cells are grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl culture medium per well, according to the media needs of the cells, in a humidified atmosphere (e.g. 37°C, 6.5% CO_2). The incubation period of the cell cultures depends on the particular experimental approach and on the cell line used for the assay. For most experimental setups, the incubation of cells for 24 to 96 h is appropriate.
2	After the incubation period, add 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well.
3	Incubate the microplate for 4 h in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
4	Add 100 µl of the Solubilization solution into each well.
5	Allow the plate to stand overnight in the incubator in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
6	Check for complete solubilization of the purple formazan crystals and measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

3.2 Examples

3.2.1. Cell growth assay procedure

Additional reagents required

- Culture medium, e.g. DMEM containing 10% heat inactivated FCS (fetal calf serum), 2 mM glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine-monohydrate, 50 µM 2-mercaptoethanol, HT-media supplement (1×), containing 0.1 mM hypoxanthine and 16 µM thymidine. If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin or gentamicin *
- Interleukin-6, human (hIL-6) (200000 U/ml, 2 µg/ml) sterile*.

Protocol

For the determination of human interleukin-6 (hIL-6) activity on 7TD1 cells (mouse-mouse hybridoma) (see fig. 4).

Step	Action
1	Seed 7TD1 cells at a concentration of 2×10^3 cells/well in 100 µl culture medium containing various amounts of IL-6 (final concentration e.g. 0.1–10 U/ml (0.001–0.1 ng/ml)) into microplates (tissue culture grade, 96 wells, flat bottom).
2	Incubate cell cultures for 4 days at 37°C and 6.5% CO_2.
3	After the incubation period, add 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well.
4	Incubate the microplate for 4 h in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
5	Add 100 µl of the Solubilization solution into each well.
6	Allow the plate to stand overnight in the incubator in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
7	Check for complete solubilization of the purple formazan crystals and measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

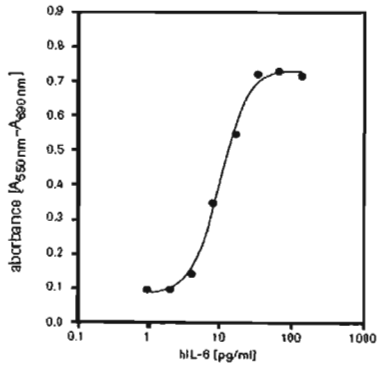

Fig. 4: Proliferation of 7TD1 cells (mouse-mouse hybridoma) in response to recombinant human interleukin-6 (hIL-6) using the procedure described (see section *Examples*, 3.2.1).

3.2.2 Cytotoxicity assay procedure

Additional reagents required

- Culture medium, e.g. RPMI 1640 containing 10% heat inactivated FCS (fetal calf serum), 2 mM glutamine and 1µg/ml actinomycin C_1 (actinomycin D). If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin or gentamicin*
- Tumor necrosis factor-α, human (hTNF-α) (10 µg/ml)*, sterile*.

Protocol

For the determination of the cytotoxic effect of human tumor necrosis factor-α (hTNF-α) on WEHI-164 cells (mouse fibrosarcoma) (see fig. 5).

Step	Action
1	Preincubate WEHI-164 cells at a concentration of 1×10^6 cells/ml in culture medium with 1 µg/ml actinomycin C1 for 3 h at 37°C and 6.5% CO_2.
2	Seed cells at a concentration of 5×10^4 cells/well in 100 µl culture medium containing 1 µg/ml actinomycin C_1 and various amounts of hTNF-α (final concentration e.g. 0.001–0.5 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom).
3	Incubate cell cultures for 24 h at 37°C and 6.5% CO_2.
4	After the incubation period, add 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well.
5	Incubate the microplate for 4 h in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
6	Add 100 µl of the Solubilization solution into each well.
7	Allow the plate to stand overnight in the incubator in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
8	Check for complete solubilization of the purple formazan crystals and measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

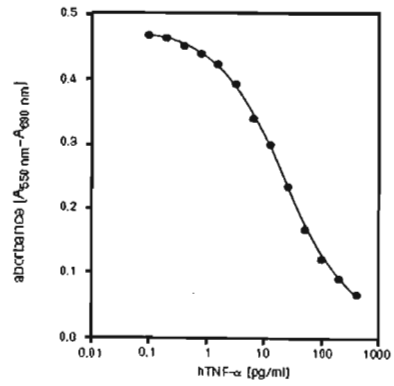

Fig. 5: Determination of the cytotoxic activity of recombinant human TNF-α (h TNF-α) on WEHI-164 cells (mouse fibrosarcoma) using the procedure described (see section *Examples*, 3.2.2).

3.2.3. Assay procedure for the analysis of neutralizing monoclonal antibodies to growth factors or cytokines

Additional reagents required

- Culture medium, e.g. RPMI 1640 containing heat inactivated 10% FCS (fetal calf serum), 2 mM L-glutamine. If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin or gentamicin*.
- hGM-CSF (10 000 U/ml, 1 µg/ml), sterile *
- anti-hGM-CSF (200 µg/vial), lyophilized, sterile.

Protocol

For the determination of the inhibitory activity of a murine, monoclonal antibody to human granulocyte-macrophage colony stimulating factor (anti-hGM-CSF) on hGM-CSF activity on TF-1 cells (human erythroleukemic cells).

Note: Recombinant, human interleukin-3 (hIL-3)*, which also is effective on TF-1 cells, can be used as a negative control (see fig. 6).

Step	Action
1	Preincubate culture medium containing hGM-CSF (5 U/ml, 0.1 ng/ml) and various amounts of anti-hGM-CSF (final concentration e.g. 0.01–50 µg/ml) in microplates (tissue culture grade, 96 wells, flat bottom).
2	Add TF-1 cells at a concentration of 5×10^3 cells/well in 50 µl culture medium and incubate for 48 h.
3	After the incubation period, add 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well.
4	Incubate the microplate for 4 h in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
5	Add 100 µl of the Solubilization solution into each well.
6	Allow the plate to stand overnight in the incubator in a humidified atmosphere (e.g. 37°C, 6.5% CO_2).
7	Check for complete solubilization of the purple formazan crystals and measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

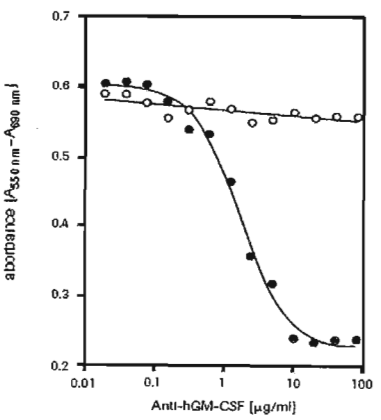

Fig. 6: Inhibition of recombinant human GM-CSF (5 U/ml; 0.1 ng/ml) (●), but not recombinant human interleukin-3 (0.4 U/ml; 0.2 ng/ml) (○) activity on TF-1 cells (human erythroleukemic cells) by anti-hGM-CSF (clone 3092) using the procedure described (see section *Examples*, 3.2.3.).

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Note: A reference list for microtiter tetrazolium assays (e.g. MTT, XTT, WST-1) is available on request.

Related Products

Parameter	Detection by	Product	Cat. No.
BrdU labeling of proliferating cells	*In situ* assay	• BrdU Lab. and Det. Kit I • BrdU Lab. and Det. Kit II • *In Situ* Cell Proliferation Kit, FLUOS	11 296 736 001 11 299 964 001 11 810 740 001
	ELISA	• BrdU Lab. and Det. Kit III • Cell Proliferation ELISA, BrdU (colorimetric) • Cell Proliferation ELISA, BrdU (chemiluminescent)	11 444 611 001 11 647 229 001 11 669 915 001
	Single reagents for *in situ* assays and ELISA applications	• Anti-BrdU* formalin grade • Anti-BrdU -FLUOS, formalin grade • Anti-BrdU -Peroxidase, Fab fragments, formalin grade • FixDenat	11 170 376 001 11 202 693 001 11 585 860 001 11 758 764 001
Measurement of metabolic activity	Quantification in microplate	• Cell Proliferation Kit I (MTT) • Cell Proliferation Kit II (XTT) • Cell Proliferation Reagent WST-1	11 465 007 001 11 465 015 001 11 644 807 001

* available from Roche Applied Science

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www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country. Country-specific contact information will be displayed. Use the Product Search function to find Pack Inserts and Material Safety Data Sheets.

Roche Diagnostics GmbH
Roche Applied Science
68298 Mannheim
Germany

APPENDIX G: Preparation of the incubation buffer (25ml)

140mM Nacl =58.44

C=n/v

N= 0.0028 moles

N=m/M

Mass = 0.0028 X 58.44

= 0.1636g or 164mg

5mM $CaCl_2$ = 174.02

C=n/v

N=cv

= 0.005M X 0.02L

= 0.0001

Mass = nM

= 0.001 X 147.02

= 0.01472g or 14.70mg

10mM Hepes buffer

$C_1 . V_1 = C_2 . V_2$

C_1 = 1M

V_1 = x

C_2 = 0.01M

V_2 = 25ml

X = 0.01. 25/1

= 0.25ml or 250µl of Hepes

. Make up to 25ml

Nacl and $CaCl_2$ are weighted out into 25ml volumetric flask and 250ul of Hepes is added and made up into a mark with deionised water.

APPENDIX H: Annexin-v- and PI assay

For life science research only. Not for use in diagnostic procedures.
FOR *IN VITRO* USE ONLY.

Annexin-V-FLUOS Staining Kit

Kit for the detection and quantification of apoptosis and differentiation from necrosis at single cell level, based on Annexin-V-labeling

Cat. No. 11 858 777 001 50 tests
Cat. No. 11 988 549 001 250 tests

Version May 2005
Store at +2 to +8°C

1. Product overview

Kit contents

Vial/ Cap	Label	Content/Cat.No.		Use
		11 858 777 001	11 988 549 001	
1 green	Annexin-V-Fluorescein	110 µl	500 µl	Ready-to-use
2 red	Propidium Iodide	150 µl	500 µl	• Ready-to-use • For the preparation of the Annexin-V-Fluorescein labeling solution
3 blue	Incubation buffer	50 ml HEPES buffer	4 × 50 ml HEPES buffer	• Ready-to-use • For the dilution of the Annexin-V-Fluorescein solution

Introduction

In the early stages of apoptosis, changes occur at the cell surface (1, 2, 3). One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell (4). Fadok et al. showed that macrophages specifically recognize PS exposed on the surface of lymphocytes during the development of apoptosis (2). The recognition and phagocytosis of apoptotic cells and bodies protects organisms from the exposure to cellular compounds leading to inflammation, which mostly accompanies necrosis.

Assay principle

The analysis of phosphatidylserine on the outer leaflet of apoptotic cell-membranes is performed by using Annexin-V-Fluorescein and Propidium Iodid (PI) for the differentiation from necrotic cells or labeling with a cell surface marker for cell characterization.
The procedure involves:

Stage	Description
1	Washing the cells in PBS.
2	Incubation of cells with Annexin-V-Fluorescein in a Hepes buffer containing PI or labeling reagent for cell surfaces (*e.g.*, CD-marker).
3	Analysis of the samples under a fluorescence microscope or on a flow cytometer.

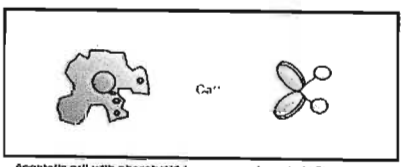


Fig. 1: Test principle.

Application

Annexin V is a Ca^{2+} -dependent phospholipid-binding protein with high affinity for phosphatidylserine (4). This protein can hence be used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane and is therefore suited to detect apoptotic cells (4, 5, 6, 7) in cell populations but not on tissue sections. Since necrotic cells also expose PS according to the loss of membrane integrity, apoptotic cells have to be differentiated from these necrotic cells. The simultaneous application of a DNA stain which is used for dye exclusion tests allows the discrimination of necrotic cells from the Annexin V positively stained cell cluster. Any other secondary labeling should be possible, *e.g.*, membrane surface staining with a phycoerythrin or TRITC-labeled monoclonal antibody for further cellular characterization (8).

Sample material

- Cell lines
- Freshly isolated cells

Number of tests

For 50 tests (Cat. No. 11 858 777 001)
For 250 tests (Cat. No. 11 988 549 001)

Preparation

Recombinant Annexin- V is produced in *E coli* (strain NB42). The GST-tagged protein is purified by standard purification protocols.

Fluorescence characteristics

Annexin-V-Fluorescein and propidium iodide show the following fluorescence characteristics:

	Fluorescein	Propidium Iodide
Excitation	488 nm	488–540 nm
Emission	518 nm	617 nm

Specificity

Annexin-V-Fluos binds in a Ca^{2+}-dependent manner to negatively charged phospholipid surfaces and shows high specificity to phosphatidylserine. Therefore, it stains apoptotic as well as necrotic cells. Propidium Iodide stains DNA of leaky necrotic cells only.

Storage/Stability

Stable at +2 to +8°C until the expiration date printed on the label.

2. Procedures and required material

Additional solutions required

PBS*

Preparation of Annexin-V-FLUOS labeling solution

Predilute 20 µl Annexin-V-Fluos labeling reagent (Vial 1) in 1 ml Incubation buffer (bottle 3) and add 20 µl Propidium Iodide solution (Vial 2).
Note: 1 ml is enough for 10 samples.

Staining of cell suspensions

In the following table please find the staining procedure for cell suspensions.

Step	Action
1	Wash 10^6 cells with PBS and centrifuge cells at 200 × *g* for 5 min
2	Resuspend the cell pellet in 100 µl of Annexin-V-FLUOS labeling solution. Incubate 10–15 min at 15–25°C.
3	Analyze by fluorescence microscopy or on a flow cytometer (see 3. Analysis)

Staining of adherent cells

In the following table please find the staining procedure for adherent cells.

Step	Action
1	Remove chambers and silicon borders of cells grown on chamberslides.
2	Remove medium and cover slides with Annexin-V-FLUOS labeling solution (100 µl/chamber).
3	Put coverslips on slides and incubate for 10–15 min at 15–25°C.
4	Analyze by fluorescence microscopy or on a flow cytometer (see 3. Analysis). ***Note:*** We do not recommend to analyze adherent cells by flow cytometry, because trypsinization or scraping for monodispersion of the cells results in false positive staining and analysis of non-dispersed cell clusters.

3. Analysis

Fluorescence-microscopy

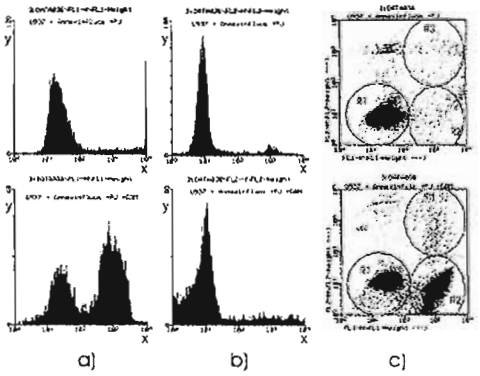
For evaluation by fluorescence microscopy use an excitation wavelength in the range of 450–500 nm (*e.g.*, 488 nm) and detection in the range of 515–565 nm (green).

Flowcytometry

Add 0.5 ml incubation buffer (bottle 3) per 10^6 cells and analyze on a flow cytometer using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter >600 nm for PI detection. Electronic compensation of the instrument is required to exclude overlapping of the two emission spectra. Typical histograms of apoptotic versus non-apoptotic and necrotic cells are shown in figure 2.

Figure 2

FACS analysis of apoptotic U937 cells after staining with Annexin-V-FLUOS and propidium iodide. Cells were then stained with the components of the Annexin-V-FLUOS Staining Kit and analyzed. Cultivation for 4 h in the presence (lower row) or absence (upper row) of 4 µg/ml camptothecin:
a) single parameter Annexin-V-FLUOS,
b) single parameter propidium iodide and
c) dual parameter (FL1 – Annexin-V-FLUOS; FL2 – propidium iodide): Cluster R1 –living cells, R2 – apoptotic cells and R3 – necrotic cells.



x-axis = increasing Annexin-V-fluorescence [relative light unit (rlu)]
y-axis = increasing Propidium Iodide (rlu)
Result: Flow cytometric analysis clearly differentiates normal (living) cells with low Annexin and low PI staining, apoptotic cells with high Annexin and low PI staining, and necrotic cells with high Annexin and high PI staining.

4. References

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5. Related products

This table only shows a selection of the most important products related to the product described in this pack insert.
For further information please access our Apoptosis special interest site:
http://www.roche-applied-science.com/apoptosis

Apoptosis-specific physiological change	Detection mode/ Product	Pack size	Cat. No.
DNA fragmentation	**Gel Electrophoresis**		
	Apoptotic DNA-Ladder Kit	20 tests	11 835 246 001
	***In situ* assay**		
	In Situ Cell Death Detection Kit, TMR red	1 kit (50 tests)	12 156 792 910
	In Situ Cell Death Detection Kit, Fluorescein	1 kit (50 tests)	11 684 795 910
	In Situ Cell Death Detection Kit, AP	1 kit (50 tests)	11 684 809 910
	In Situ Cell Death Detection Kit, POD	1 kit (50 tests)	11 684 817 910
	Single reagents for TUNEL and supporting reagents		
	TUNEL AP	70 tests (3.5 ml)	11 772 457 001
	TUNEL POD	70 tests (3.5 ml)	11 772 465 001
	TUNEL Enzyme	2 × 50 µl (20 tests)	11 767 305 001
	TUNEL Label Mix	3 × 550 µl (30 tests)	11 767 291 910
	ELISA		
	Cell Death Detection ELISA	1 kit	11 544 675 001
	Cell Death Detection ELISA^{PLUS}	1 kit (96 tests)	11 774 425 001
	Cell Death Detection ELISA^{PLUS}, 10×	1 kit	11 920 685 001
	Cellular DNA Fragmentation ELISA	1 kit (500 tests)	11 585 045 001
Cell membrane alterations	**Microscopy or FACS**		
	Annexin-V-Alexa 568	250 tests	03 703 126 001
	Annexin-V-Biotin	250 tests	11 828 690 001
	Annexin-V-FLUOS	250 tests	11 828 681 001
	Annexin V FLUOS Staining Kit	50 tests 250 tests	11 858 777 001 11 988 549 001
Enzymatic activity	**Western Blot**		
	Anti-Poly (ADP-Ribose) Polymerase	100 µl	11 835 238 001
	FIENA		
	Caspase 3 Activity Assay	1 kit	12 012 962 001
	Fluorimetric microplate Assay		
	Homogeneous Caspase Assay, fluorometric	100 tests 1000 tests	03 005 372 001 12 236 869 001
	***In situ* Assay**		
	M30 CytoDEATH (formalin grade)	50 tests 250 tests	12 140 322 001 12 140 349 001
	M30 CytoDEATH, Fluorescein	250 tests	12 156 857 001
Expression of apoptosis-related proteins	**Apoptosis Induction**		
	Anti-Fas (CD95/APO-1)	1000 tests	11 922 432 001
	ELISA		
	p53 pan ELISA	1 kit	11 828 789 001

** available from Roche Applied Science*

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Use the Product Search function to find Pack Inserts and Material Safety Data Sheets.

Inspiring Discovery

Roche Diagnostics GmbH
Roche Applied Science
Nonnenwald 2
82372 Penzberg
Germany

APPENDIX I: Actinomycin D

3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 · (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

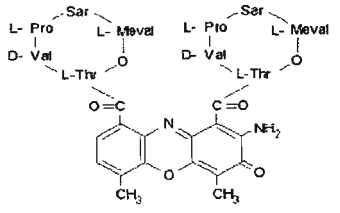
ProductInformation

Actinomycin D
from *Streptomyces* sp.

Product Numbers **A 1410, A 4262, A 9415, A 5156**
Storage Temperature 2-8 °C

CAS NUMBER: 50-76-0

Synonyms: Dactinomycin; Actinomycin IV; Actinomycin C_1



Molecular formula: $C_{62}H_{86}N_{12}O_{16}$
Molecular weight: 1255.42

Melting point: decomposes at 241.5-243 °C [1]
$E_{1\%}$ (244nm) = 281 (methanol) [1]
$E_{1\%}$ (441nm) = 206 (methanol) [1]
E_{mM} (240 nm) = 34.1 (methanol) [2]
E_{mM} (443 nm) = 24.4 (methanol) [2]
$[\alpha]^{25}_{D}$: –315° (c = 0.25% in methanol) [1]

Product Description
Actinomycin D is an antineoplastic antibiotic that inhibits cell proliferation. It is a cytotoxic inducer of apoptosis against tumor cells.[3] The compound inhibits the proliferation of cells in a nonspecific way by forming a stable complex with double-stranded DNA (via deoxyguanosine residues), thus inhibiting DNA-primed RNA synthesis. It also causes single-strand breaks in DNA.[4,5,6]

Actinomycin D has been shown to be an inhibitor of the minus-strand transfer step in reverse transcription [7,8] and therefore is used in studying and suppressing HIV-replication. It has also been shown to suppress programmed cell death of PC12 cells induced by etoposide, an inhibitor of topoisomerase II. [9]

It is used in cell culture as a selection agent. Actinomycin D binding to yeast in ribosomal RNA has been studied.[10] The sensitivity of various strains of E.coli to Actinomycin D and the mechanism of binding has been studied.[11,12] The structure was determined by atomic structure and amino acid sequence.

Actinomycin D is an antibiotic used for its antineoplastic properties in the treatment of various malignant neoplasms including Wilm's tumour, and the sarcomas. Adverse effects include bone marrow depression and gastrointestinal toxicity; it is extremely irritating and extravasation produces severe tissue damage. [4]

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Actinomycin D is sold as red shiny crystals and in solution it is a clear liquid. Sigma tests solubility of A1410, A4262, A9415 in acetonitrile or acetone at 10 mg/mL. It is soluble in DMSO at a minimum of 1 mg/mL. Several references [4,5,13] claim slight solubility in water (about 0.5 mg/mL).

Storage/Stability
The powder is hygroscopic and sensitive to light. When stored sealed and protected from light and moisture, at 2-8 °C, Actinomycin D remains unchanged (as tested by HPLC) for at least 15 months.

Dilute solutions of Actinomycin D are very sensitive to light.[1] This product tends to adsorb to plastic and glass on standing in solution.[13] For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution are expected to be stable for at least a month at –20 °C.

A1410 Actinomycin D 98%
Lyophilized powder with a purity of 98% (HPLC)

A4262 Actinomycin D 95%
Lyophilized powder with a purity of 95% (HPLC)

A9415 Actinomycin D,
cell culture tested
Lyophilized powder with purity of 95% (HPLC). For cell culture applications, actinomycin D is used as a selection agent and is used in banding techniques to differentiate between different regions of chromosomes. Suggested working concentrations is 1 µg/mL.

A5156 Actinomycin D-Mannitol
Lyophilized powder containing 1 mg of actinomycin D (98% purity) and 49 mg of Mannitol. It is soluble in water at 20 mg/ml.

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APPENDIX J: Cycloheximide

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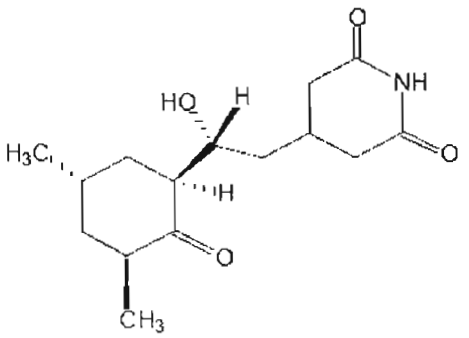
ProductInformation

CYCLOHEXIMIDE
From a microbial source

Product Number **C7698 and C1988**
Storage Temperature 2-8 °C

CAS# 66-81-9
Synonyms: Actidione, Naramycin A, (3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide)

Product Description



Molecular Formula: $C_{15}H_{23}NO_4$
Molecular Weight: 281.4

Cycloheximide is a glutarimide antibiotic derived from a microbial source. It inhibits eukaryotic, but not prokaryotic protein synthesis. This selective inhibition makes it active against many yeasts and fungi, but tolerated by most bacteria. Classically, it has been used in bacteriological media to isolate or count bacteria in the presence of yeast and molds (100–1000 µg/ml).

At the cellular level, cycloheximide blocks the translation of messenger RNA on cytosolic, 80S ribosomes, but does not inhibit organelle protein synthesis.[1,2] This biochemical action makes cycloheximide a powerful tool in the study of many cellular processes. For example, studies of cells pretreated with cycloheximide (1-20 ug/ml) and untreated cells have helped determine the role of protein synthesis in apoptosis,[4,5,6] gene expression,[7,8,9] and steroidogenesis.[2,10]

Cycloheximide is also used in plant research. It stimulates ethylene production in fruit and leaves.[11,12]

Preparation Instructions
Cycloheximide has been reported to be soluble up to 2% (w/v) in water (20 mg/ml).[4] The powder will dissolve slowly in water and this process may be aided by mixing or sonication. More dilute solutions (5mg/ml) may be prepared without sonication. Aqueous solution stability is pH dependent. Solutions are stable for several weeks in the pH range of 3 to 5, which is the range optimal for stability. For longer term solution storage, refrigeration is suggested. Solutions refrigerated over 18 months, retained 75% of the original activity.[3] Solutions prepared in distilled water may be sterile filtered or autoclaved without serious loss of activity.[3] Autoclaving of medium containing cycloheximide is not recommended. The sterile cycloheximide solution should be added to sterile broth or cooled, sterile, melted agar.[3] Boiling for one hour at pH 7 destroys activity, but no loss of activity is observed after 15 minutes. Under acidic conditions (pH 2), activity is not destroyed after one hour of boiling.[4] Under dilute alkali conditions, decomposition occurs rapidly in solution at room temperature with the formation of 2,4-dimethylcyclohexanone.[4]

Cycloheximide is also soluble in most organic solvents, including ethanol, with the exception of saturated hydrocarbons.[4]

Storage/Stability
Store desiccated at 2-8 °C. The product as supplied should be stable for 5 years if properly stored.

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