Geographical Information System for spatial microbiological data analysis in pharmaceutical manufacturing

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed:_____

Christian Pruckner

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Abbreviations and Technical Terms

APA	Aseptic Processing Area (grades A/AB/B) where the pharmaceuticals are filled in their final containers and if required lyophilised
As-built	Cleanroom condition where the installation is complete, but no production equipment, materials or personnel present
Aseptic processing area :	Cleanroom for aseptic processing
At-rest	Cleanroom condition where the installation is complete with equipment installed and operating or operational, but no personnel present
Bioburden:	The total number of microorganisms associated with a specific item (product, surface or water)
Biocontamination:	Contamination of surfaces, liquids, materials, persons or air with viable particles
Biodecontamination:	Removal of microbial contamination or reduction to an acceptable level
CAD:	Computer Aided Design - computer system to assist in the creation, modification or analysis of a design
CAPA:	Corrective and Preventive Actions – project tool for quality improvement
cfu:	colony forming unit
cGMP	current Good Manufacturing Practice
Critical Processing Zone	Grade A area where the critical operations e.g. filling and stoppering of drugs or sterile filtration connection take place
Change Control:	Formal process used to ensure that changes to a product or system are introduced in a controlled and coordinated manner
CIP	Cleaning in Place
Cleanroom:	Room environment with controlled level of environmental particles specified by the number of particles per cubic meter at a specified particle size (different values for each cleanroom grade)
EM	Environmental Monitoring – a monitoring system established for the cleanroom environment
EU:	European Union

GA:	General Area - rooms with no cleanroom validated status deviations during production e.g. alert level excursions
GDP:	Good Documentation Practises
GIS:	Geographic Information Systems
GMP:	Good Manufacturing Practise
Grade:	EU GMP classification of cleanrooms
HEPA filter:	High Efficiency Particulate Air filter to control level of environmental particles
In-operation	Condition where the installation is complete with equipment installed, operating with a specified number of personnel present and working
ISO:	International Organization of Standardization
Management review:	Presentation to senior management about the production status, hot topics and issues for allocation of resources
Media-fill	Aseptic process simulation study using a microbiological growth medium
m/s	meters per second
NCA:	Non Classified Area - includes building areas such as staircases
NCR:	Non-Conformance Report. A quality tool to investigate
Non-viable particles:	non-living particles
Pa	Pascal
primary packaging material	Vial, syringe or other “primary” packaging material containing the drug
QRM	Quality Risk Management
Riboprinting:	Molecular technology tool for the study of genetic diversity in microorganisms
Std. Dev.:	Standard Deviation
Sterile Filling Suite:	Grade A area in which the pharmaceuticals are filled and the surrounding environments AB/B
SIP	Sterilisation in Place
SQL*LIMS	Structured Query Language Laboratory Information Management System
spp.	species (plural)
ULPA filter	Ultra-Low Penetration Air filter
USP	United States Pharmacopeia
Viable particles:	Living particles - microorganisms and their spores

1 Abstract

Microbiological data analysis and trending in pharmaceutical cleanrooms is a legislative requirement, and part of quality assurance. It has historically been conducted using statistical tools, such as control charting, which lack the geographic component. Three years of environmental monitoring data from the cleanrooms of a pharmaceutical facility were analysed to find cleanrooms with microbiological percentage recovery rate hot spots in the air and surface of the facility. The data set was evaluated to determine if it has changed over the three year period using a χ^2 analysis. The cleanroom microflora was analysed to see if it differs among the different cleanroom grades, and potential contamination routes in the cleanrooms were ascertained. Given the lack of published studies that use GIS to perform spatial analysis of microbiological environmental monitoring data, this approach is novel. The research concluded that areas which are highly frequented by personnel (connection corridor, vial washing areas, documenting rooms, and air locks) have higher percentage recovery rates, and some are microbial hot spots. The microbiological percentage recovery rates from air and surface monitoring in some of these and other areas have improved over the three year period, while in some cleanroom areas they have not. The microbial percentage recovery rates from air monitoring have improved more than those from surface monitoring over the three year period. The percentage recovery rates in most cleanrooms, however, have remained stable over the three year period. The microbial distribution within Grade D and the Aseptic Processing Area (cleanroom grades A, A2 and B) during the three year period is very similar in terms of microbial class distribution and microbial species identification. Grade D, however, has a higher percentage of moulds and a lower number of Gram-positive rods without spores compared with the Aseptic Processing Area. The material and personnel flow within the facility is responsible for the majority of microbiological contamination of the cleanrooms. This correlates with the findings of the literature review.

2 Introduction and Literature Review

Geographic Information Systems (GIS) is not simply a blend of applications, rather it is a research exploration tool in itself. GIS facilitates a holistic analysis of geographic issues and provides the framework to deal with a large number of attributes in data (Schuurman, 1999). GIS is often essential to understand what is happening and what is going to happen in the geographic space which allows the stipulation of precise actions and it improves communication between disciplines (GIS.com, n.d.). John Snow's cholera maps from the 19th century, well known in epidemiological circles, showed the power of mapping in data analysis two centuries ago. Managed care providers, hospital administrators, and especially pharmaceutical companies are just beginning to utilize GIS as an effective means to improve their organisational processes and services (Lang, 2000). This advanced approach to managing geographic data is transforming the way organisations operate (GIS.com, n.d.).

While there are multiple uses of GIS in many disciplines and at small scale, the literature review did not reveal any research about the use of GIS in a pharmaceutical cleanroom environment to analyse environmental monitoring data spatially. Although, there are programs available that support pharmaceutical cleanroom data analysis using statistics (especially control charting) e.g. Quality Analyst® from Northwest Analytics (2013), and programs that provide simple graphical depiction of the cleanroom and its attribute data e.g. MODA™ Paperless QC Micro Solution (LONZA, n.d.), see Figure 1 on page 2, these software packages lack proper spatial analysis tools. This makes the application of spatial analysis for cleanroom environmental data using GIS software novel, and it makes this research a contribution to the current knowledge. Furthermore, the application of spatial analysis could become an additional quality tool for current Good Manufacturing Practices (cGMP) and quality assurance utilised in pharmaceutical manufacturing.

The first part of the literature review focuses on subject-related small scale, microbiological and epidemiological uses of GIS. The second part outlines the pharmaceutical manufacturing process focussing on Environmental Monitoring (EM) and applicable legislative requirements, thereby reflecting on the pharmaceutical facility used in this study.

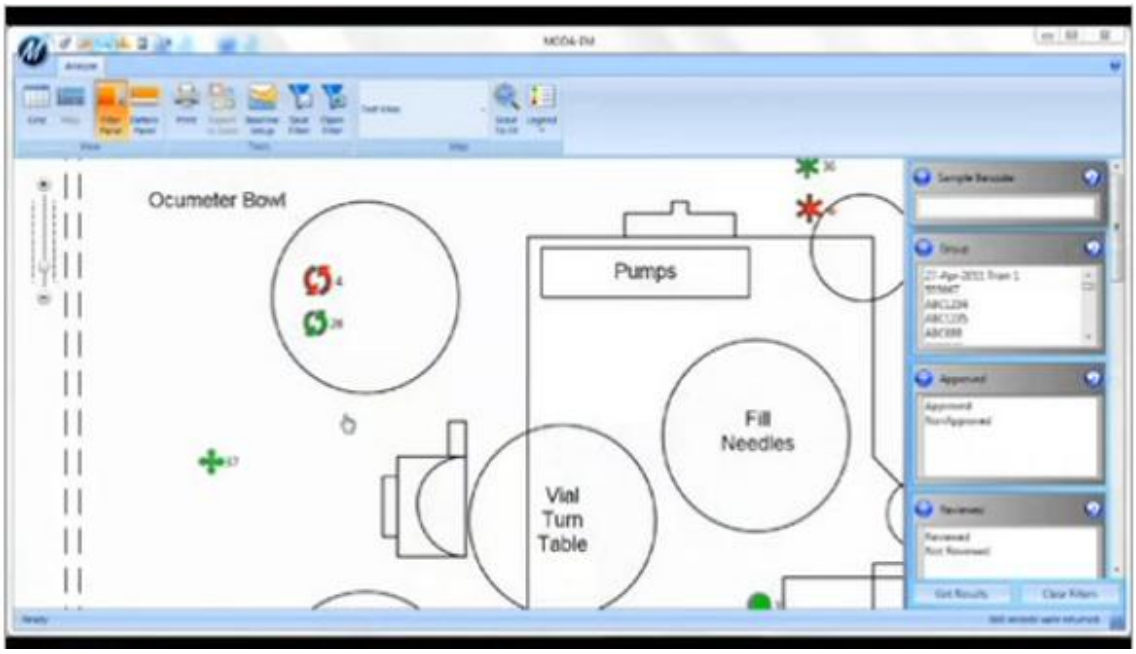

Figure 1: Simple graphical depiction of the cleanroom and its attributes by MODA™ Paperless QC Micro Solution (LONZA, n.d.)

2.1 Small scale, microbiological and epidemiological uses of GIS

GIS can be used at most scales, from large to small, although its main purpose has been broad-scale data analysis. The discrete object conceptualisation pictures the world as a table-top surface, with discrete, countable items. Discrete objects can be represented depending on size as points, lines, polygons, and volumes. This conceptualization is appropriate when dealing with buildings (Goodchild, 2011).

ArcGIS 10 software has been used in the hospital environment of the UK Chandler Hospital Pavilion A to detail each room about occupancy, accompanying assets, and technologies (see Figure 2 on page 3). It included the integration of CAD drawings, 360° panoramic pictures of rooms, an asset editing module, and web site linking (Bossard, 2010). Campus Basemap (an ArcGIS Map Document) was utilized to create a multi-scale high resolution basemap for a business and university campus. This basemap could be used for many different purposes. It can be a basis for a diversity of desktops, mobile and web mapping applications to orient map users and support areas such as facility management. The basemap can then be combined with other map layers that contain operational information from the user. The operational layers may be interior spaces within the buildings or additional infrastructure management. The basemap allows the overlay of interior spaces and assets within buildings, and the source data in the geodatabase is kept in its local State Plane Projection (Esri Inc., 2012).

Figure 2: Custom GIS application of Chandler Hospital Pavilion (Bossard, 2010)

A high resolution GIS (Hospital GIS) was used by Kistemann et al. (2000) to investigate a nosocomial *Salmonella* outbreak at a university hospital. The GIS was fed with case-specific data of each *Salmonella* infection case, and a "*Salmonella*" outbreak layer, comprising the data of each case (patients carrying Salmonella symptomatically or asymptomatically), was created and added to the already established layers "placement of institutions" and "logistics" comprising buildings, wards, diagnostic and therapy areas, central kitchen, central sterilisation and technical networks (spatial data). The cases were georeferenced to their assumed place of infection (e.g. workplace, ward etc. See Figure 3 on page 4).

The attribute data of the "*Salmonella*" outbreak layer included the microbiological examination of food and surface samples from the hospital kitchen and nosocomial infections, as well as stool samples from affected persons. Furthermore, the data from diagnostic facilities, transport facilities, technical facilities, food supply, therapeutic facilities, sterilisation methods, disinfection methods and staff duty roster were also assigned as attributes. Epidemiological queries were performed using the GIS for every case and single functional units for spatio-temporal relationships in relation to the outbreak. A meal (pudding) contaminated with salmonella through poultry manipulation in close proximity during pudding production in the hospital kitchen was identified as the most likely source of the outbreak. The use of GIS proved to be invaluable in this epidemiological outbreak investigation (Kistemann et al., 2000).

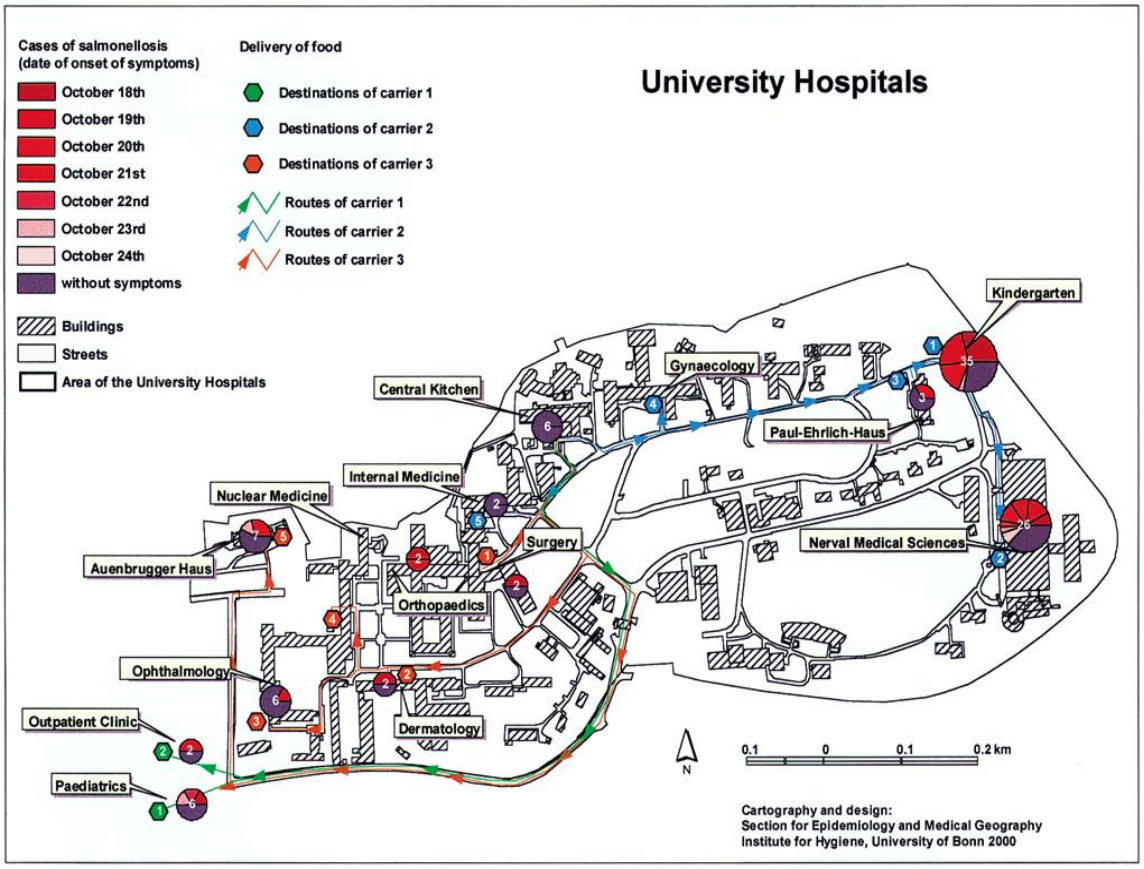

Figure 3: Temporal-spatial distribution of symptomatic Salmonellosis cases within the hospital campus and food delivery system (Kistemann et al., 2000)

Lamar (2003) used GIS and riboprinting technology to examine potential relationships between farm animals and their environments by monitoring transmission of bacterial pathogens (*Salmonella, Campylobacter jejuni and E. coli* O157:H7) on the dairy farm environment and to a neighbouring waterway (river). The study consisted of an epidemiological study analysing more than 40,000 environmental and animal samples for the chosen bacterial pathogens. GIS was used in this study to pinpoint critical sources of contamination at the farm and to identify the vehicles by which those particular bacteria pathogens were spread amongst the animals (Lamar, 2003).

An aerial photograph taken of the farm was scanned into ArcView® GIS version 3.2 and used as basemap onto which the gathered spatial information (microbiological results) was layered. The number of identified bacterial pathogens isolated at each location of the dairy farm were sorted, grouped quarterly per annum and plotted on the basemap. Advanced statistical analysis of the spatial data was performed including cluster analysis and Poisson probability distributions using the ArcView® GIS program. The study concluded that *Salmonella* (32%) was the most prevalent isolated pathogen followed by *C. jejuni* (21%) and *E. coli* O157:H7 (2%). Insect and bird droppings, feeding, bedding and water were identified as significant vectors of transmission. The

microbiological and GIS analysis revealed high prevalence of Salmonella in the river at and upstream of the dairy farm but not *C. jejuni* and *E. coli* O157:H7. However, the farm could not be identified as significant contributor of pathogens to the river (Lamar, 2003).

Understanding the spatial relationships and patterns of infectious diseases aids in finding the causes and implementing preventive controls. GIS and remote sensing are increasingly used to analyse geographic distribution of diseases and the relationship between pathogenic factors and the geographic environment. Analytical and even basic applications of GIS in the field of epidemiology can help in analysing and visualizing the geographic distribution of vectors and diseases through time, thereby exposing relationships, patterns, and spatio-temporal trends which would be difficult or impossible to detect otherwise. GIS helps with outbreak investigation, spatial spread, and dynamics. GIS can also aid in the coordination of responses to the issue at hand for prevention and control measures (Ruankaew, 2005).

As an example, GIS has been used to map the geographic distribution of annual human deaths from rabies in Thailand. The GIS analysis included a spatial-temporal depiction of annual human deaths from rabies using choropleth maps and overlays of layers, such as the railroad network and other georeferenced information. The analysis revealed that the overall death toll decreased from 1994-2003 and also that there seemed to be a relationship between high frequency rabies affected areas and transportation routes, see Figure 4 on page 5 (Ruankaew, 2005).

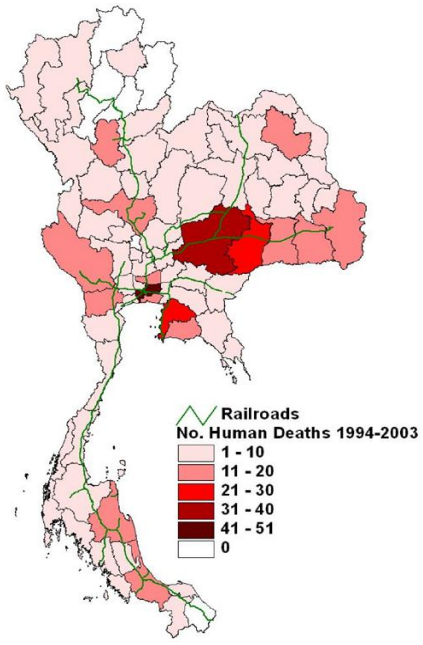

Figure 4: Geographic distribution of human deaths from rabies in 1994-2003 with the location of railroads (Ruankaew, 2005)

2.2 Pharmaceutical manufacturing

There are a range of compliance obligations for the manufacture of medical devices and pharmaceuticals stated in GMP guidelines, ISO-Standards, technical documents, pharmacopoeias, etc. (ISO 13408-1, 2008). Drug manufacturing for New Zealand is governed by the New Zealand Code of Good Manufacturing Practice for Manufacture and Distribution of Therapeutic Goods, which is a compilation of international GMP guidelines (New Zealand Medicines and Medical Devices Safety Authority, 2009). New Zealand, however, does not have its own pharmacopoeia for the manufacture of pharmaceuticals (World Health Organisation, 2011). Pharmaceutical companies operating within the EU require manufacturing authorisations from the EU whether the products are sold within or outside the Union (European Commission, 2010). To obtain and maintain a manufacturing authorisation, a pharmaceutical manufacturing facility requires a comprehensively designed and implemented Pharmaceutical Quality System based on Quality Risk Management (QRM) and GMP. QRM consists of all the organised arrangements to ensure that the manufactured pharmaceuticals and medical products are of the required quality for their intended use, while GMP ensures that drugs are consistently manufactured and controlled to the set quality standards. An overview of a typical QRM process can be seen in Figure 5 on page 7. Quality Control (QC), a part of GMP, is concerned with specifications, sampling, and testing, along with the documentation, organisation, and release of products that have passed the quality review (European Commission, 2013, a).

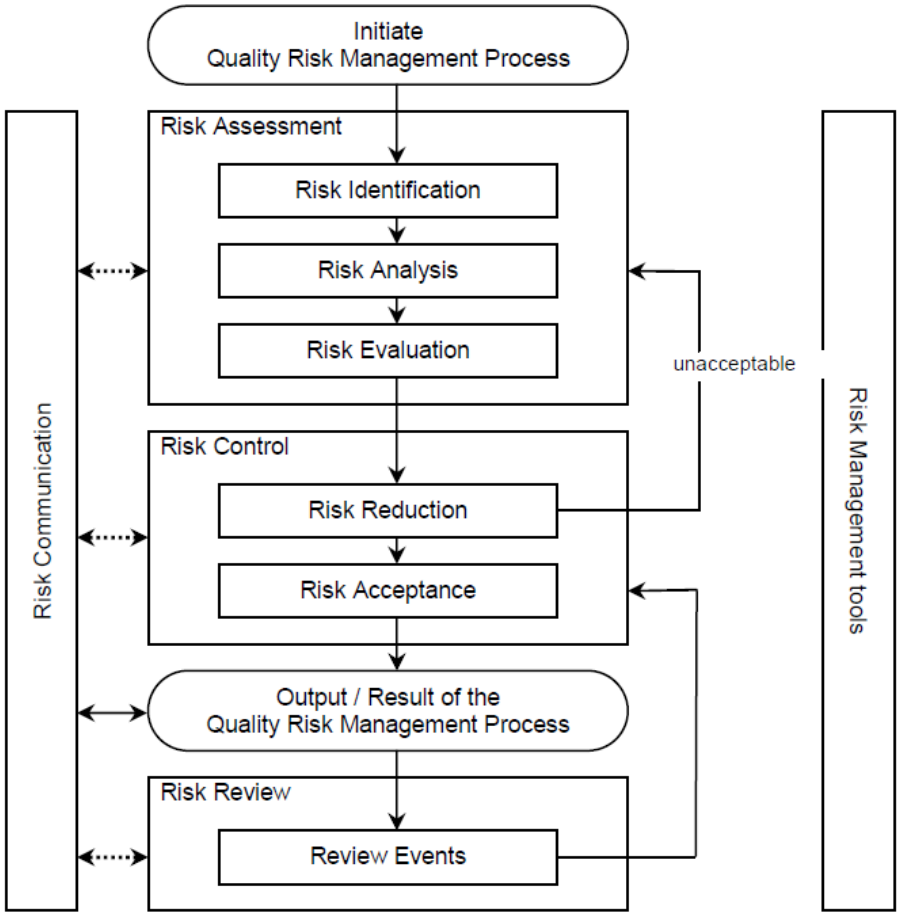

Figure 5: Quality risk management process (Pharmaceutical Inspection Convention, 2009/b)

Pharmaceutical manufacturing is governmentally regulated. Laws that govern these activities, e.g. Code of Federal Regulations (for USA), etc., are disseminated in the industry. Auditors such as the US Food and Drug Administration validate that these laws and standards are put into action (Arter, 1994). Manufacturing sterile products necessitates particular requirements to minimise risks for the product through particulate, pyrogen and microbiological contaminations (European Commission, 2008). Pharmaceuticals in general and especially drugs for injection or intravenous application must achieve compendial limits according to the applicable pharmacopeia for particulate matter and sterility (The United States Pharmacopeial Convention, 2012/a). Quality Assurance (QA) with validated methods for processes must be stringent for this type of pharmaceutical manufacturing. The manufacturing processes of sterile drugs are categorised by those where the product is terminally sterilised and those where each stage or all stages are achieved aseptically (European Commission, 2008). The latter process is employed by the pharmaceutical manufacturing facility used in this study at all stages to manufacture sterile products. The sterilisation of pharmaceuticals in their final containers, although the safer alternative, is not always

possible due to negative effects on the drugs from the sterilisation procedures. (ISO 13408-1, 2008).

Sterility is the absence of microorganisms which cannot be assured through testing of the final container. Instead, sterility of the product is assured through a suitable manufacturing process with a validated sterilisation filtration step at the end just before aseptic filling of the product into its final container in a cleanroom environment. For this, a compendial Sterility Assurance Level (SAL) has to be achieved. This is the degree of assurance that a specified process renders a population of final containers sterile. The SAL is also the probability of non-sterile final containers in that population. The SAL for final containers is 10^{-6} which equates to a probability of no more than one viable microorganism in $1x10^6$ sterilised final containers (European Directorate for the Quality of Medicines & HealthCare, 2011/a).

2.3 Cleanroom environment

A cleanroom is an environment in which the concentration of particles is controlled and the room is constructed and utilised in a way to minimise the generation, introduction and retention of particles (viable and non-viable) (Whyte, 2010). Cleanroom environments are of enormous value in the manufacture and assembly of many industrial products, including medical devices, pharmaceuticals, electronics, and spacecraft components (La Duc et al., 2007). Cleanrooms must be smooth, impervious, and undamaged, to reduce the accumulation and shedding of micro-organisms as well as other particles, and to allow an efficient cleaning/disinfection of these areas. The grading of cleanrooms depends on the maximum concentration limits (particles/m^3 of air) and size of particles equal to and larger than the specified sizes in Table 1 on page 9. The non-viable particle sizes that are monitored in the pharmaceutical industry are 0.5µm and 5µm (Whyte, 2010). Cleanrooms are controlled according to physical parameters, including temperature, humidity, differential pressure, air velocity (for laminar air flows), viable (microorganisms), and non-viable particle counts (through the use of High Efficiency Particulate Air filters (HEPA) or Ultra Low Penetration Air (ULPA) filters (Concept Heidelberg, 2004; Tierney, Burke, O'Donnell, & McAteer, 2010).

Non-viable particles equal to and larger than the specified sizes (≥0.5µm and ≥5µm) are counted using calibrated discrete particle-counting light-scattering instruments. Sampling locations for all listed parameters are evenly distributed throughout the cleanroom based on an initial risk analysis of the process. Environmental monitoring (EM) measurements are performed in the vicinity of the exposed materials during

periods of activity based on this risk assessment. All the parameters are monitored as part of the EM-programme for which the testing results of each cleanroom or cleanroom zone must be recorded and checked for compliance with the specified alert- and action levels as well as for negative trends in the data (European Commission, 2008; Health Canada, 2009; ISO 14644-1, 1999; ISO 14644-2, 2000; Tierney et al., 2010). Alert levels are defined to indicate a drift from normal conditions, while action levels are established to control the environment; they trigger immediate root-cause investigation and implementation of corrective and preventive actions if exceeded (ISO 14698-1, 2003).

Table 1: Non-viable maximum concentration limits and particles sizes in cleanroom classes

ISO classification	**Maximum concentration limits (particles/m^3 of air) and size of particles equal to and larger than the specified sizes.**					
Particle size	≥0.1µm	≥0.2µm	≥0.3µm	≥0.5µm	≥1.0µm	≥5.0 µm
ISO 1	10	2				
ISO 2	100	24	10	4		
ISO 3	1000	237	102	35	8	
ISO 4	10,000	2370	1020	352	83	
ISO 5	100,000	23,700	10,200	3520	832	29
ISO 6	1,000,000	237,000	102,000	35,200	8320	293
ISO 7				352,000	83,200	2930
ISO 8				3,520,000	832,000	29,300
ISO 9				35,200,000	8,320,000	293,000

(Whyte, 2010)

Table 2: Correlation between cleanroom grades in USP, EU GMP Guide, FDA GMP Guide, ISO 14644-1, ISO 13408-1 with the required viable and non-viable particle action levels as well as the levels implemented by the pharmaceutical facility used in this study (yellow columns) (Concept Heidelberg, 2004; European Commission, 2008; ISO 13408-1, 2008; ISO 14644-1, 1999; The United States Pharmacopeial Convention, 2012/a)

European Commission EU GMP guide (Annex 1)	FDA GMP guide	ISO 14644-1	ISO 13408-1	USP35 <1116>	Pharmaceutical study facility	Processes	Non viable particles				Non viable particles				Non viable particles				Non viable particles			
							≥ 0,5 µm m^3		≥ 5 µm m^3		≥ 0,5 µm m^3		≥ 5 µm m^3		≥ 0,5 µm m^3		≥ 5 µm m^3		≥ 0,5 µm ft^3 (m^3)		≥ 5 µm ft^3 (m^3)	
							in operation	at rest	in operation	at rest	in operation	at rest	in operation	at rest	in operation	at rest	in operation	at rest	in operation	at rest	in operation	at rest
A	100	5	Critical processing zone	5	A	Isolator closesd (sterile)	3520	3520	20	20	3520	n.a.	n.a.	n.a.	3520	n.a.	n.a.	n.a.	99 (3500)	99 (3500)	0 (1)	0 (1)
A	100	5	Critical processing zone	5	A	Aseptic formulation and filling	3520	3520	20	20	3520	n.a.	n.a.	n.a.	3520	n.a.	n.a.	n.a.	99 (3500)	99 (3500)	0 (1)	0 (1)
A	100	5	Critical processing zone	5	A2	Transfer aseptic area where product is partially sealed	3520	3520	20	20	3520	n.a.	n.a.	n.a.	3520	n.a.	n.a.	n.a.	99 (3500)	99 (3500)	0 (1)	0 (1)
B	10,000	7	Direct support zone	7	A2	Aseptic packaging where the product is completely closed in this area	352,000	3520	2900	29	352,000	n.a.	n.a.	n.a.	352,000	n.a.	n.a.	n.a.	9912 (350,000)	99 (3500)	57 (2,000)	0 (1)
B	10,000	7	Direct support zone	7	B	Enviroment for clean room areas of aseptic formulation and filling	352,000	3520	2900	29	352	n.a.	n.a.	n.a.	352,000	n.a.	n.a.	n.a.	9912 (350,000)	99 (3500)	57 (2,000)	0 (1)
B	10,000	7	Direct support zone	7	B	Air locks	352,000	3520	2900	29	352	n.a.	n.a.	n.a.	352,000	n.a.	n.a.	n.a.	n.a.	99 (3500)	n.a.	0 (1)
A	100	5	Critical processing zone	5	Grade A Air Zone	Tranfer area/enviroment in LA24/B after lyophilisation till crimping (product closed with stoppers)	3520	3520	20	20	3520	n.a.	n.a.	n.a.	3520	n.a.	n.a.	n.a.	99,122 (3,500,000)	99 (3500)	566 (20,000)	0 (1)
C	100,000	8	Indirect support zone	8	C and critical zone D	Open production processes after plasma pooling and thawing until pharmaceutical formulation before sterilization filtration, air locks	3,520,000	352	29	2900	3,520,000	n.a.	n.a.	n.a.	3,520,000	n.a.	n.a.	n.a.	99,122 (3,500,000)	9912 (350,000)	566 (20,000)	57 (2,000)
D	not defined	not defined	not defined	not defined	D	Plasma pooling and thawing, closed production processes, handling, washing and storage of production equipment, air locks	not defined	3,520,000	not defined	29	not defined	n.a.	n.a.	n.a.	not defined	n.a.	n.a.	n.a.	100,000 (3,531,000)	99,122 (3,500,000)	747	566 (20,000)

European Commission EU GMP (Annex 1)	FDA GMP guide	ISO 14644-1	ISO 13408-1	USP35 <116>	Pharmaceutical study facility	Processes	Viable Air control cfu/m3 in operation	Viable Air control cfu/m3 at rest	Viable Air control cfu Settle plate	Viable Air control cfu/m3 in operation	Viable Air control cfu/m3 at rest	Viable Air control cfu Settle plate	Viable Air control cfu/m3 in operation	Viable Air control cfu/m3 at rest	Viable Air control cfu Settle plate	Surface control cfu / plate in operation	Surface control cfu / plate at rest	Surface control cfu / plate in operation	Surface control cfu / plate at rest	Surface control cfu / plate in operation	Surface control cfu / plate at rest	Personnel control cfu/plate in operation	Personnel control cfu/plate in operation	Personnel control cfu/plate in operation
A	100	5	Critical processing zone	5	A	Isolator closesd (sterile)	<1	n.a.	< 1 / 4h	1[1]	n.a.	1/4h[1]	<0.1%	n.a.	<0.1%	0	0	0	< 1	n.a.	<0.1%	n.a.	0	0	n.a.	n.a.	<0.1%
A	100	5	Critical processing zone	5	A	Aseptic formulation and filling	< 1	n.a.	< 1 / 4h	1[1]	n.a.	1/4h[1]	<1%	n.a.	<1%	< 1	< 1	< 1 / 4h	< 1	n.a.	<1%	n.a.	1/plate & max. 60%[2]	1/plate & max. 60%[2]	<1 (glove print 5 fingers cfu/glove)	1 / plate ∑max 1 (5 finger / plate)[3]	<1%
A	100	5	Critical processing zone	5	A2	Transfer aseptic area where product is partially sealed	<1	n.a.	<1 / 4h	1[1]	n.a.	1/4h[1]	<1%	n.a.	<1%	1	< 1	1 / 4h	< 1	n.a.	<1%	n.a.	(b)	(b)	<1 (glove print 5 fingers cfu/glove)	5/plate - hands, sleeves, mask and goggles	<1%
B	10,000	7	Direct support zone	7	A2	Aseptic packaging where the product is completely closed in this area	10	n.a.	5 / 4h	10	n.a.	5 / 4h	<1%	n.a.	<1%	1	< 1	1 / 4h	5	n.a.	<5%	n.a.	(b)	(b)	5 (glove print 5 fingers cfu/glove)	5/plate - hands, sleeves, mask and goggles	<5%
B	10,000	7	Direct support zone	7	B	Enviroment for clean room areas of aseptic formulation and filling	10	n.a.	5 / 4h	10	n.a.	5 / 4h	<5%	n.a.	<5%	3	< 1	n.a.	5	n.a.	<5%	n.a.	Surface 3/ floor 5	1/plate & max. 60%[2] floor 3	5 (glove print 5 fingers cfu/glove)	5/plate (hands/ sleeves/ mask/ googles) 10/plate (overall / hood)	<5%
B	10,000	7	Direct support zone	7	B	Air locks	(a)	n.a.	5/ 4h	10	n.a.	5/ 4h	<5%	n.a.	<5%	7	< 1	n.a.	5	n.a.	<5%	n.a.	Surface 4/ floor 5	(a)	5(glove print 5 fingers cfu/glove)	only for cleaning personnel as for grade B	<5%
A	100	5	Critical processing zone	5	Grade A Air Zone	Tranfer area/enviroment after lyophilisation before crimping (product closed with stoppers)	<1	n.a.	<1 / 4h	1[1]	n.a.	1/4h[1]	<1%	n.a.	<1%	n.a.	< 1	n.a.	<1	n.a.	<1%	n.a.	10	1/plate & max. 60%[2]	<1 (glove print 5 fingers cfu/glove)	n.a.	<1%
C	100,000	8	Indirect support zone	8	C and critical zone D	Open production processes after plasma pooling and thawing until pharmaceutical formulation before sterilization filtration, air locks	100	n.a.	50/ 4h	100	n.a.	50/ 4h	<10%	n.a.	<10%	88	9	n.a.	25	n.a.	<10%	n.a.	surface 10/ floor 20	surface 10/ floor 15	not defined	15 (5 Finger/ sleeves) (d)	<10%
D	not defined	not defined	not defined	not defined	D	Plasma pooling and thawing, closed production processes, handling, washing and storage of production equipment, air locks	200	n.a.	100/ 4h	not defined	n.a.	not defined	not defined	n.a.	not defined	200	55	n.a.	50	n.a.	not defined	n.a.	50	20	not defined	n.a.	not defined

[1] Samples from Class 100 (ISO 5/Grade A) enviroments should normally yield no microbiological contaminants according to the US GMP Guide

[2] If more than 1 agar plate is used for a single sampling point (e.g. floor) then only 60% of plates may have microbial growth (e.g. 6 out of 10 plates)

[3] Only 1 hand or forearm may have 1 cfu/plate

There are different classifications for cleanrooms, and different terms used for cleanroom classifications, according to GMP guides, ISO standards and pharmacopoeias, of which the most prominent are listed in Table 2 on page 10, which also shows the required viable and non-viable particle limits, as well as the limits implemented by the pharmaceutical facility used in this study. This study uses the European grading system via alphabetic capital letters A-D to refer to cleanroom classifications. Cleanrooms are differentiated by a cascading barrier and airlock systems with particle and microbial counts starting at Grade A, this being the cleanest environment where final containers are being sterile filled and stoppered, also called the “critical zone” according to ISO 13408-1 (2008). This Grade A zone can be surrounded by a Grade B cleanroom area that serves as a zone in which in-process materials, equipment and containers/closures are aseptically prepared, held or transferred, and where personnel in cleanroom gowning are located. Grade A and B combined is called the Aseptic Processing Area (APA) according to the FDA Aseptic Guide (Concept Heidelberg, 2004).

Grade A is the critical zone because the processed drugs are vulnerable to contamination during the filling and stoppering process. This is why controlling the number of particles (viable and non-viable) is paramount to avoid potential contamination of the product. Air monitoring samples in Grade A should normally yield no microbiological contaminants. Grade C and Grade D are cleanroom areas where the products are manufactured prior to sterilization filtration and where the less critical logistical parts of the operation are executed, hence higher particle and microbial counts are expected. Grade D areas are often used for equipment cleaning, e.g. vials before they enter the APA through a dry heat sterilisation tunnel. Grade A and supporting cleanroom areas associated with it are of particular importance to sterile drug production. The pharmaceutical manufacturing (filling) facility in this study also has a cleanroom grade that cannot be found in any guideline or legislative script called Grade A2; this is a mixture of grades A and B in regard to action limits for viable and non-viable particles and physical parameters where the environmental cleanroom conditions are maintained at Grade A requirements, but the action limits in terms of viable and non-viable particles are less stringent, and closer to Grade B limits depending on the cleanroom use (see Table 2 on page 10). In addition, there is another cleanroom grade called “Grade A Air Supply” which is a designated area used for the crimping of the final container vials under laminar air flow when this cannot be performed in Grade A of the APA (Concept Heidelberg, 2004; European Commission, 2008; ISO 13408-1, 2008; Pharmaceutical Inspection Convention, 2009/b; Tierney et al., 2010).

The air within cleanrooms moves from areas of higher cleanroom grade and thus lower particle counts to adjacent lower cleanroom grades thereby maintaining a positive pressure differential of at least 10-15 Pascal (Pa) designed to maintain the air quality within the different cleanroom zones. Another air quality maintenance tool is the air exchange rate of at least 20 air changes per hour in Grade D and higher rates in higher grades (e.g. 40 air changes per hour). Plastic curtains and rigid plastic shields are among the barriers used to achieve segregation of the aseptic processing line often times separating Grade A from Grade B. Materials of construction within cleanrooms ensure ease of cleaning and sanitizing, such as rounded floors to wall junctions, and generally smooth and hard durable surfaces. Cleanrooms generally do not contain unnecessary fixtures, materials or equipment and equipment does not obstruct or disturb air flow (Concept Heidelberg, 2004).

There are two types of ventilation systems utilised in the cleanroom environment, namely those with unidirectional air flow and those with non-unidirectional (turbulent) air flow. The Grade A and sometimes Grade B cleanroom environments have an unidirectional air flow, often called laminar air flow, where a high air flow volume is directed either horizontally or vertically across the wall or ceiling after it passes a HEPA or ULPA filter (Whyte, 2010). HEPA or ULPA filters are required to be leak tested on a regular basis; at least twice a year in the APA (ISO 14644-1, 1999; ISO 14644-2, 2000). The velocity of the unidirectional air flow is usually between 0.3-0.5 m/s, or 0.45 m/s in the case of the cleanroom environment of this study. The air sweeps through the room and exits either through the floor or through outlets at the bottom of the walls, thereby extracting airborne contaminants from the room. The lower cleanroom classes of grades C, D and sometimes B, however, have often a non-unidirectional air flow where lower air volumes are dispersed within the room, mainly from the ceiling, after the air passes a HEPA or ULPA filter. For non-unidirectional air flows, the incoming air is mixed with the room air and then extracted through outlets, as in the case of unidirectional air flow. The air exchange rate is usually ≥20 per hour. The unidirectional air flow is much more efficient in extracting air contaminants and is therefore utilised in Grade A (and Grade A2 in the case of this study) (Whyte, 2010).

Personnel and material flow procedures into cleanrooms, especially in the APA, are specified and restricted to trained qualified personnel in order to:

- maintain the integrity of the critical cleanroom environment
- minimise the entry and retain contaminants from outside/ancillary areas into the APA
- prevent cross-contamination within the APA and to ensure segregation of

sterilised and non-sterilised equipment/components

Materials taken into critical APA are sterilised except in justified cases where sterilisation is not possible (e.g. with microbiological air sampling devices) in which case the equipment must be biodecontaminated in material air locks or H_2O_2/peracetic acid chambers into the APA. Furthermore, equipment should be kept in the APA in order to minimise the risk of introducing contaminants. Sterilising and biodecontamination procedures for materials used in the APA must be validated according to the applicable standards and validation plans (ISO 13408-1, 2008).

The components of the primary packaging material are sterilised separately and the finished vial or syringe is assembled in an aseptic manner. Aseptic processing requires a sound knowledge of different sterilisation procedures. For instance, glass vials or syringes are subjected to dry heat, rubber stoppers are subjected to moist heat, and the liquid drug product is subjected to a sterilisation filtration process. Any manual or mechanical manipulation of the sterilised container, closure, drug, or component prior or during aseptic assembly poses a contamination risk and hence requires vigilant control. Therefore, each critical manufacturing step necessitates validation and quality assurance (Concept Heidelberg, 2004; The United States Pharmacopeial Convention, 2012/d). Cleaning-in-place (CIP) and Sterilisation in Place (SIP) are the methods used for cleaning and sterilising the internal surfaces of sections of equipment or entire process systems without - or with only minimal - disassembly. This method allows for validated and automated cleaning and sterilisation, mainly of surfaces with direct product contact, and is often used to ensure segregation and sterility of tanks, pipes, hoses, and parts that contain the product, e.g. the pipe and tanks from the sterile filter to the filling needles in Grade A (ISO 13408-4, 2004; ISO 13408-5, 2006).

Water used in pharmaceutical manufacturing is treated to various levels of purity depending on its intended use. Different water purity classifications in ascending order of purity include treated source water, purified water (PW), and water for injection (WFI). Treatment methods can include ozonation, chlorination, reverse osmosis, filtration, heat treatment, and distillation. These treatments are intended to remove ions and to reduce the bioburden in the water to a limit required for its purpose. Purified water in fixed systems is circulated in a loop to reduce the formation of biofilms inside tanks and pipes. In addition, WFI must be loop-circulated and maintained at >70 degree C to inhibit the growth of common microorganisms. The different water classes must also be frequently monitored for total organic carbon (TOC), conductivity, water bioburden and endotoxins. The monitoring must be documented for all points of use where the water can be drawn from the loop (ISO 13408-1, 2008). A trending of the

different parameters drawn from the points using spatial analysis of the whole loop and the points of use can be useful in the investigation of deviations especially in regard to biofilm formation leading to an increase of water bioburden and potentially increased endotoxin levels.

2.4 Aseptic processing

Aseptic processing is defined as the handling of sterile materials in a controlled environment in regard to air supply, equipment design, facility materials used and trained personnel to control viable (microbial) and non-viable particulate contaminations to acceptable levels (Lampe, 2013). Aseptic processing covers all production steps following product and component sterilisation until the final container is sealed. The sterile drugs are processed in a controlled cleanroom environment where viable and non-viable particle levels are maintained at defined levels to pose minimal risk. It is essential that sources of contamination from product, components, materials, facility, equipment, personnel and utilities including water systems are considered and minimised (ISO 13408-1, 2008; The United States Pharmacopeial Convention, 2012/a). Aseptic processes are designed to minimize hazards of contaminant exposure to sterile articles in the manufacturing operation by providing the highest possible environmental controls, optimising process flows, and designing equipment (Concept Heidelberg, 2004). Microbiological risk management strategies are essential in aseptic processing and follow the four stages:

- Identification of contamination risks, including origins, routes, microbial proliferation, contamination, and adequate removal of microbes (initial risk assessment).
- Assessment of contamination risks on product quality using monitoring data and process evaluation. For this, risk minimisation measures should consider the initial risk assessment.
- Monitoring and detection of contamination using appropriate tools including isolation and identification of microbial contaminants (environmental monitoring).
- Correction and prevention of contamination such as additional training, procedural modification or design changes as well as effectiveness checks of the implemented measures.

(ISO 13408-1, 2008).

There are different technologies used in aseptic processing and filling of drugs including:

- Isolators – enclosed chambers where drugs are processed or filled and which can be decontaminated before use, e.g. by H_2O_2. Isolators are currently the state-of-the-art technology for aseptic processing (Agalloco, 2005).
- Restricted Access Barrier Systems (RABS) – filling suites with Grade A that may only be accessed in-operation by personnel through thick glove and sleeve assemblies (intervention gloves) attached to the walls surrounding Grade A
- Blow/fill/seal technology where the final container is formed, filled and closed in a continuous operation
- Conventional cleanroom filling lines with Grade A processing and filling environments that may be accessed by personnel in-operation through intervention gloves or direct access

The latter technology is used in this study. Conventional cleanroom filling lines which can be accessed by operators have a higher contamination risk due to personnel intervening in Grade A in-operation compared with RABS and isolators. However, RABS are only state-of-the-art if the doors are kept close and isolator gloves are exclusively used to intervene in Grade A. While the other technologies substantially reduce the contamination risks and have found acceptance in the industry, the conventional cleanroom filling line technology was the industry standard for many years (Agalloco, 2005) and is still widely used in pharmaceutical manufacturing (The United States Pharmacopeial Convention, 2012/a). In spite of this, isolators and blow/fill/seal technology are the future of aseptic processing and will replace conventional cleanroom filling lines which will result in obtaining Sterility Assurance Levels (SALs) similar to those achieved by terminal sterilisation (Madsen, 2003).

In addition, conventional cleanroom filling lines are outdated and no longer conform with cGMP according to Lysfjord (2012, June). This is also reflected in the number of warning letters issued by the FDA to pharmaceutical companies using conventional filling lines compared with those relying on isolator technology. This is why the FDA is placing heightened scrutiny on pharmaceutical companies still using this older technology. This means that these companies have the burden of more frequent audits by authorities. Moreover, these companies have to provide convincing proof and justification to consistently produce sterile products. (Lysfjord, 2012, June).

Equipment used in aseptic processing including sterilizers, filter assemblies, filling machines, etc., must be qualified through validation to ensure its suitability for the

intended purpose. Specified user requirements and qualification of materials and equipment, including design, installation, operation, and performance in the APA are paramount to ensure optimal aseptic manufacturing. Furthermore, processes carried out with specified equipment are re-qualified at regular intervals (ISO 13408-1, 2008).The aseptic processing and filling operations must be validated and regularly revalidated by the use of microbiological growth medium (e.g. CASO bouillon) instead of the product. The medium is used instead of the real product to simulate the storage, transport and aseptic filling of the product after sterile filtration, to ascertain and prove that the process is under control and the product remains sterile during processing. For this validation, also known as “media run” or “media-fill”, the filled and sealed containers with the medium are incubated to detect microbial contamination and approve, qualify and clear the process and filling line for the actual products (Concept Heidelberg, 2004).

Each change to a line or product must be evaluated and risk-assessed by the means of a written change control system (Concept Heidelberg, 2004). For this, written procedures are required to illustrate the actions to be taken if a change is proposed to a method of production, process equipment, product component, process environment or site, testing, starting material, or any other change that may affect the reproducibility of the process and product quality. Change control procedures ensure that adequate supporting data are created to show that the modified process will continue to produce a product of the desired consistent quality and within approved specifications (European Commission, 2001). Since changes are often assessed by different bodies within an organisation, understanding the change is paramount for the risk assessments. This is why a graphical display of the change on the e.g. filling line using a GIS or CAD program can be a very useful. A GIS tool has the additional benefit of better overlaying the before and after drawings of the change.

The use of double-door or integrated steriliser or decontamination units (e.g. autoclave, peracetic acid, H_2O_2) ensures validated sterilisation or decontamination and direct product flow from a lower to higher cleanroom grade. Airlocks with interlocking doors facilitate improved control of contaminants originating from materials and personnel throughout the processing facility. Airlocks are generally installed between the aseptic manufacturing area and adjoining unclassified areas or areas of lower cleanroom grade. It is crucial to adequately control material and personnel flow from lesser to higher classified cleanroom areas to prevent/reduce the influx of contaminants (Concept Heidelberg, 2004; European Commission, 2008; Pharmaceutical Inspection Convention, 2009/b). However, microbial contaminants are inevitable in any cleanroom

environment where operators are present because of the natural shedding of microorganisms by humans (The United States Pharmacopeial Convention, 2012/a). In this regard, written procedures should address how material and personnel are to be introduced into the aseptic processing rooms (Concept Heidelberg, 2004).

2.5 Contamination factors

Improper control of the critical factors within the cleanroom may pose a risk to product quality and the cleanliness of the cleanroom. Viable and non-viable particulate contamination factors in the cleanroom include:

- Cleanroom gowning
- Personnel
- Facility design and stationary equipment
- Materials, portable and mobile equipment
- Cleanroom cleaning
  (ISO 14644-5, 2004).
- The air within cleanrooms
  (The United States Pharmacopeial Convention, 2012/a).

2.5.1 Cleanroom gowning

Personnel scatter particles from their skin and non-cleanroom garments. The amount of particles varies, but can be up to several million particles per minute with hundreds of microorganism-carrying particles (ISO 14644-5, 2004), given that, the human skin can host up to 1×10^6 microorganisms per cm^2 (Hall, Mackintosh, & Hoffman, 1986). The cleanroom garment's primary function is to act as a barrier that protects processes and the drugs from human contamination (ISO 14644-5, 2004). For this, gowns must be sterilized (for the APA), low particle-emitting, covering the skin along with the hair, and providing a barrier between the body and exposed sterilised materials, thereby preventing contamination through viable and non-viable particles shed from the body. This is why the fabric of cleanroom gowns is designed to reduce particle dispersion by the carrier (Concept Heidelberg, 2004). Cleanroom garments must completely envelope the carrying person, with good closures at the neck, ankles and wrists. Cleanroom undergarment (underneath the cleanroom garment) can additionally reduce particle dispersion by personnel. However, even fully gowned personnel showing no exposed skin will still shed low numbers of microorganisms (Sandle, 2011). In addition, although the majority of non-viable and viable particles originate from the skin and non-cleanroom clothing, non-viable and to a lesser extent viable particles (if garments are not sterilised for grades C and D, for example) are also disseminated from the surface of cleanroom garment fabrics. The selection of cleanroom garment for personnel varies

according to the process requirements along with cleanroom grade. Humans emit microbe-carrying particles also from the mouth through talking, sneezing and coughing. This is why cleanroom garments, hair nets, beard nets and safety glasses should be worn by personnel in grades C and D. In addition, face masks, hoods and gloves may be necessary to reduce contaminants in grades C and D (ISO 14644-5, 2004).

Cleanroom garments in the APA normally include overalls, overboots, hoods, face masks, goggles and gloves (ISO 14644-5, 2004). Personnel working in the APA wear fully enclosed protective clothing that sheds virtually no particulate matter or fibres, and retains non-viable and viable particles emitted from the body and undergarments. However, a complete retention of particles cannot be assured, as the garments are not airtight and movement of the operators will exude particles. To reduce this contamination risk, personnel working in any cleanroom - especially in the APA - must follow the code of conduct within the cleanrooms (Health Canada, 2009).

2.5.2 Personnel movement and conduct

Personnel is the most important factor and greatest source of microbial contamination within the cleanroom, especially during manual aseptic processing in any pharmaceutical manufacturing operation (Lampe, 2013). It is impossible to maintain quality assurance for manufacturing without properly trained and educated employees. Studies indicate that humans constantly shed viable and non-viable particles even with their cleanroom gowning on. The contamination risk in the cleanroom environment correlates with the movement of personnel within the cleanroom (The United States Pharmacopeial Convention, 2012/a). According to Lampe (2013), human performance failures and deviations are linked to:

- The continuous time duration during which personnel carry out repetitive aseptic activities – the operator's aseptic technique can deteriorate with the passage of time. (this is why media-fills are designed to cover the worst case in time duration to simulate this constraint)
- The frequency of the activities
- Complex aseptic processing tasks
- Personnel fluctuation

Conventional filling lines require that both material and personnel flows are optimised to prevent unnecessary activities that could increase exposure of the product, production equipment, or primary packaging material to contaminants. For this, the layout of the facility and equipment must provide for ergonomics that optimise processes and movement of operators. The flow of personnel must be designed to limit

the frequency of entries and exits to aseptic cleanrooms and especially interventions in the critical Grade A area (Concept Heidelberg, 2004; ISO 13408-1, 2008), and a maximum occupancy number of personnel in any cleanroom should be set regardless of grade (ISO 14644-5, 2004). Furthermore, only the minimum number of personnel should be present within the cleanrooms at any one time, and the set number of personnel in the cleanrooms of the APA must not be exceeded. (Health Canada, 2009). Any stoppage or intervention during an aseptic process may increase the risk of contamination. Due to the interconnection of the various cleanrooms within a processing facility, it is essential to carefully define and control the dynamic interactions required between cleanrooms, especially of those with different grades e.g. movement of material between cleanroom grades (Pharmaceutical Inspection Convention, 2009/a).

Since people generate particles, it is vital that only essential personnel enter cleanrooms hence, access must be restricted, documented, and enforced. Untrained personnel and visitors should not enter the cleanroom unless it is unavoidable. If this is the case, information and training about personnel hygiene, prescribed gowning procedures and prescribed behaviour within the cleanroom must be provided to the visitors (Pharmaceutical Inspection Convention, 2009/a). Training for cleanroom personnel must include elements of aseptic practices, sterility assurance, gowning practices, sterilisation (Lampe, 2013), correct gowning, gowning change procedures, hygiene, discipline, conduct, and safety, as well as basic elements of microbiology. Rigorous discipline and strict supervision is paramount in order to maintain environmental quality, and personnel training is critical, especially for staff working in the APA. Furthermore, training for sampling personnel is also important, as inappropriate sampling methods could contaminate critical areas within the APA (Concept Heidelberg, 2009; European Commission, 2008, 2013, b; ISO 14644-5, 2004; Reich, Miller, & Patterson, 2003; The United States Pharmacopeial Convention, 2012/a). In fact, Madsen (2003) points out that the sensitivity of the EM methods has improved as technology advanced to the point that in some incidences the act of sampling may introduce more contaminants to the processing environment than what may have existed before.

In addition, the effectiveness of the training must also be periodically assessed (Pharmaceutical Inspection Convention, 2009/a) and also regularly retrained. As a result of the training, operators must be able to apply classroom training by consistently excelling in aseptic gowning, assembly and technique without contaminating the product, cleanroom surfaces and their cleanroom gowning (Lampe, 2013). Additional

contamination hazards through personnel include cold, flu, dermatitis, sunburn, bad dandruff, allergic conditions (scratching, sneezing or coughing) and high microbial bioburden on personnel. Staff members suffering from these conditions must not be allowed in the cleanroom (ISO 14644-5, 2004). Any person that will have access to any area where the drug product may be exposed in the course of its manufacture, packaging, and labelling must undergo a health examination prior to employment as well as regular re-examinations based on job description. Moreover, any person with known communicable, infectious diseases or open lesions (e.g. a fresh tattoo) must not enter any area with exposed product or primary packaging material (Health Canada, 2009).

Personnel must change into cleanroom gowning before entering the cleanroom. Staff must be trained in the correct gowning procedure, initially evaluated (through personnel contact plate monitoring), re-evaluated in regular intervals (e.g. during media-fills), and retrained if action limits are exceeded (ISO 14644-5, 2004). Personnel airlocks must have a physical separation of the different phases of changing (e.g. two chamber air locks) to reduce particulate and microbial contamination of protective clothing. Personnel must wash their hands at a sink provided at the first stage of the personnel airlock (European Commission, 2008). The removal of worn and therefore contaminated cleanroom gowning must be organised in such a way so as to reduce contamination of the cleanroom, including airlocks. Furthermore, cleanroom clothing must be taken off and stored appropriately when reused (e.g. in Grade D) to avoid contamination of the airlocks and subsequently adjacent cleanrooms. Personnel operating within the cleanrooms must conduct themselves in such a way so as to minimise contamination of the cleanroom and the product manufactured/filled. Some of the conducts that should be followed include:

- Personnel should not support materials against their body to avoid any transfer of contaminants
- Talking close to the product must be reduced
- Doors should always be opened and closed slowly and never left open to reduce transfer of non-viable and viable particles between rooms and to reduce stirred-up particles
- The nose must not be blown in the cleanroom; gloves and mask must be changed in the airlock after sneezing or noise blowing
- Cleanroom gowns may be contaminated and must therefore not be touched, gloves must be disinfected in regular intervals
- Movement within the cleanroom must be slow, deliberate and methodical to avoid disrupting the air flow

- Products and equipment left standing in the cleanroom must be protected from contamination e.g. kept in a closed container or cabinet
    - To avoid dispersing particles onto process surfaces or in the product, personnel must not position themselves between clean air supplies and the process surfaces or product (ISO 14644-5, 2004)
- Personnel should never directly touch sterile components with product contact (e.g. filling needles), primary packaging material (e.g. syringes) or any surfaces within the product delivery pathway. Sterile handling equipment such as tweezers should be used instead for these tasks
  (The United States Pharmacopeial Convention, 2012/a). Then again, the assembly of the filling line may require the touching of sterilized equipment such as the filling needles. To reduce any risks from the assembly operation to the filling needles a CIP/SIP operation can be utilised after assembly.

However, although humans are a major risk factor within any cleanroom, the root cause category “human error” is often overused in CAPA investigation. This results in a lost opportunity to identify the real or underlying root cause and to reduce the probability of this root-cause reoccurring and thereby fostering continuous improvement. There are three key elements to reduce human error in regards to deviations:

- Concise and soundly-written documentation (e.g. batch records, SOPs etc.) with just enough information for the job at hand
- Effective training on those documents using a multiphase approach of continuous theoretical and practical training sessions with experienced operators who also have also the ability and training (e.g. train the trainer) to teach other people
- Monitoring performance and execution of the first two steps e.g. by using matrices that track deviation incidence rates by error code (e.g. human error) and enforces continuous improvement on documentation and training to reduce these deviations
  (Schniepp, 2013).

On the other hand, Sartain & Polarine (2011) state that although it is vital to continuously train personnel to follow rigid protocols and exercise a great deal of caution to prevent contamination of pharmaceuticals, operators still have a tendency to underestimate the impact they have on controlling a complex manufacturing process. This is due to the fact that they deal with entities too small to see, numbering in the

millions; the microbial risk is invisible and therefore often underestimated (Sartain & Polarine, 2011). To counteract this trend, the future of conventional aseptic filling lines according to Madsen (2003) may lie in the use of sealed suits in the APA, equipped with breathing air supply. This could potentially decrease operator-induced contamination in the APA to levels achieved in isolators.

2.5.3 Facility design and stationary equipment

Sound facility design that enhances the aseptic processes and reduces sources of contamination must include:

- Clear zone demarcation with interlocking doors in air locks
- Enough Heating Ventilation and Air Conditioning (HVAC) capacity to handle seasonal fluctuations in humidity and temperature
- Cleanable design
- Selection of chemical and moisture resilient materials, and
- Selection of approved cleanroom suitable materials of construction (e.g. epoxy or polymeric flooring, 316L stainless steel, etc.). However, even stainless steel may suffer from prolonged chemical exposure, resulting in pitting and rust that reduces the effectiveness of disinfectants. Drainage issues result in biofilm development which can cause persistent problems with Bacilli and other bacteria due to increased resistance to disinfectants and heat (Sartain & Polarine, 2011).

Stationary equipment includes mechanical and automated processing equipment. Bringing equipment into the cleanroom should not add contamination. Therefore, proper unpacking and cleaning/disinfecting procedures are paramount. Particle shedding materials such as cardboard must be removed before entering the cleanroom and should be vacuumed (beginning from the top to the sides) using an appropriate cleanroom vacuum cleaner with built in HEPA or ULPA filters before removal. All surfaces must be pre-cleaned/disinfected within the transfer or material airlock area. Any special equipment for lifting or moving heavy or bulky equipment should be thoroughly checked for damage and flaking surfaces, and should preferably be sterilised and bio-decontaminated (through the use of an H_2O_2 air lock), or, if these measures are not possible, cleaned/disinfected before entering the cleanroom. The wheels of carts and trolleys used to move items to or within the cleanroom must be cleaned and disinfected. Items can also be wrapped in cleanroom-compatible plastic films before being brought into the cleanroom. This method is especially of value for equipment or tools normally unsuitable for the cleanroom e.g. equipment that cannot be properly cleaned/disinfected (ISO 14644-5, 2004).

The design of the filling machine must be thorough in regard to aseptic processing, to avoid creating contamination hazards through inaccessible areas that cannot be cleaned and decontaminated properly, and to avoid unnecessary air flow turbulence created by the machine itself. Any important areas in the filling line that need frequent attention during filling should not be more than an arms-length away for aseptic interventions during filling. For this, engineering runs with e.g. WFI can be deployed to develop and refine the aseptic filling process and video recording of media-fills can be used for a detailed review intended for training or failure investigation (Lampe, 2013). Akers, Kokubo, & Oshima (2006) point out that the design of the filling line nowadays must be in such a way that eliminates interventions by personnel (even with isolator gloves) almost completely, using existing technology.

Maintenance and installation works within the cleanroom must be planned carefully. If work is scheduled during routine production time, then the rest of the cleanroom area must be isolated (e.g. through a partition). Sticky mats can be used to avoid carry-over of particles from the shoes, and neutral or negative differential pressure in the area of work should protect the adjacent cleanrooms from particles. For this, the clean-air inlet should be blocked to avoid pressurising the area of work and thus dispersing particles to other areas. Additional Environmental Monitoring (EM) is required in the surrounding cleanrooms to detect potential contaminants leaking from the area of work. After works have been completed, thorough decontamination of the affected cleanroom must take place before routine drug manufacturing can resume. Decontamination includes cleaning/disinfection, and whenever possible, sterilisation of cleanroom equipment. Fumigation of whole cleanrooms e.g. using H_2O_2 can be performed after cleanroom maintenance and production shutdowns. This aids in reducing microbiological contamination in inaccessible areas. At-rest monitoring can be performed to confirm the cleanliness status of the treated cleanrooms. Moreover, the correct performance of the cleanroom may also require re-validation before it can be used again for routing drug manufacturing especially if significant changes to the room have been made which may impact on the manufacturing process. Therefore, major maintenance work involving changes must undergo a change control process (European Commission, 2008; ISO 14644-5, 2004).

2.5.4 Materials and portable equipment

The United States Pharmacopeial Convention (2012/a) states that effective personnel and material movement is a key factor in contamination control. Personnel move equipment around the cleanrooms and they are therefore interlinked. This is why whenever possible those materials and portable equipments must be cleaned and

sterilised, or, if sterilisation is not possible, at least biodecontaminated (e.g. disinfected). In addition, ISO 14644-5 (2004) states that waste disposal (control and removal of non-cleanroom packaging) is also a significant contamination factor.

There are also time limitations that need to be considered and validated including holding sterile/depyrogenated components, sterile bulks and dirty/clean/sterile equipment (Lampe, 2013). Materials should be chosen that depending on their usage in different cleanroom grades must:

- Have tolerable anti-static properties
- Have low outgassing properties
- Be free from unwanted chemicals (e.g. organic, alkali, acid)
- Be well-suited for disinfection or sterilisation procedures
- Be free from microorganisms

Consequently, preliminary testing and/or auditing against agreed specifications is necessary for suppliers of materials used in the cleanrooms. A Supplier Corrective Actions Request (SCAR) is one of the tools used to communicate issues with materials to the supplier, both for quality improvement and to avoid further supply of non-conforming materials. Customer notification of proposed critical changes to approved materials is also required to maintain consistent quality specifications (ISO 14644-5, 2004).

Many items routinely brought in by personnel and removed from the cleanroom, such as batch records, pens, hand tools and other kinds of small portable utensils, can be a source of contaminants for the cleanroom. These items should be prevented from becoming contaminants in the non-classified area, through the use of plastic bags (or other suitable measures). This process will facilitate re-entry to the cleanroom at a later stage. The substitution of conventional paper/pen documentation with electronic means such as cleanroom-compatible computers and tablets can reduce contamination hazards. However, the cleanroom compatibility of electronic gadgets must be validated to ensure that they do not disperse viable and non-viable particles through e.g. exhausting air or keyboards that trap particles (ISO 14644-5, 2004). Furthermore, before any computerised system is put into use, it must be thoroughly tested and validated as being capable of achieving the required results. In case a current manual system is replaced by a computerised one, the two should be run in parallel for some time throughout the course of the validation (Pharmaceutical Inspection Convention, 2009/b).

2.5.5 Cleanroom cleaning

Cleanrooms are designed to be as free from contaminants as possible. Activities within the cleanroom, such as manufacturing processes, maintenance, the presence and activity of personnel, and other factors can cause contaminants to be generated and dispersed on surfaces. Therefore, frequent cleaning of all surfaces, using specified and validated cleaning operations, is necessary to prevent a risk to manufacturing processes. The cleaning process for pharmaceutical manufacturing cleanrooms requires disinfection to control the number of microorganisms. The effectiveness of the disinfectants and dilution factors used must be validated. Cleaning validation is required to confirm the effectiveness of the cleaning procedures. A rationale for selecting limits of carry-over of cleaning agents, product residues, and microbial contamination must take into consideration the materials involved (European Commission, 2001; ISO 14644-5, 2004). Some disinfectants, especially those with chlorine (often hypochlorite) may be corrosive, even to stainless steel often utilised in the cleanroom, resulting in rust and pitting, thereby reducing the effectiveness of disinfectants (Sartain & Polarine, 2011). However, the sporicidal properties of some disinfectants are necessary to control endospore-forming bacteria and mould spores (European Commission, 2001; ISO 14644-5, 2004). Furthermore, evidence must demonstrate that routine cleaning does not allow microbial proliferation, and disinfectants/sanitizers (e.g. isopropyl alcohol) must be filtered, especially in the APA, to remove microbial spores (Health Canada, 2009).

Cleaning should be avoided during manufacturing operations. Alternatively, special cleaning procedures must be in place to ensure that the product is not endangered. Some manufacturing processes may generate particles as a byproduct which must be separately contained (segregated), leaving manufacturers unable to rely on routine cleaning operations. Surfaces must be classified for the development of cleaning schedules. Critical surfaces in Grade A, where contaminants could contaminate the open product and open primary packaging materials (e.g. open vials) should be kept free of microorganisms. The vertical unidirectional airflow in Grade A helps to control the cleanliness of these surfaces. All other areas in the APA (grades B and AB) should be cleaned on a regular basis to prevent transfer of contaminants into Grade A. Personnel and material airlocks can become highly contaminated due to activities within the airlocks such as gowning of personnel and bringing in of contaminated materials. Frequent cleaning/disinfection of air locks (at least daily, or more often as required, depending on throughput) is therefore paramount to reduce the contamination levels and hinder the potential transfer of contaminants into adjacent cleanrooms of the same or higher grades (ISO 14644-5, 2004).

For all methods employed, it is paramount to redirect personnel and material traffic to allow drying of the wet surfaces and for any disinfectant to work. In Grade A cleanrooms with unidirectional flow, surfaces must only be cleaned/disinfected at-rest or after the product has been removed e.g. clearing the filling line. Similarly, cleanrooms with higher grades (A2 and B) generally should not be cleaned in-operation. Stationary equipment should be cleaned according to a risk assessment, as this equipment often contains surfaces critical to the product. Rubbish bins should be cleaned/disinfected after use, lined with plastic bags and emptied at regular intervals. Antistatic coatings can be applied to surfaces in cleanrooms to minimise static charge built up. However, antistatic coating should be even and must not flake off. Equally, anti-static surface characteristics can also be achieved simply by controlling the humidity of the air supply (ISO 14644-5, 2004).

Cleaning programmes require an understanding of the different kinds of cleanroom surfaces and the rate at which they become contaminated. Therefore, cleaning schedules must specify frequency, among other factors, such as cleaning/disinfecting agents to use for each task, and which methods to utilise, all based on a performed risk assessment for the cleanroom and operations within. An effective cleaning frequency (e.g. daily, or several times per day) means that the risk of product contamination through a contaminant transfer to critical surfaces in Grade A is reduced to an acceptable level. The entire cleanroom facility should be scheduled for thorough cleaning from top to bottom. Thorough cleaning should include service areas, storage areas, fittings, and pipes, and is best scheduled during shutdown periods. Emergency cleaning must be planned for, and may be required in events such as environmental incidents (e.g. spills, equipment failure, biological hazards), for monitoring that reveals a high occurrence of contaminants or failure of routine cleaning procedures resulting in unacceptable contamination levels. Regular at-rest monitoring after the cleaning process will verify the effectiveness of the chosen cleaning/disinfection procedures and frequencies (ISO 14644-5, 2004). More than one type of disinfectant should be used in cleanrooms of all grades to avoid a potential resistance of microorganisms to certain disinfectants (European Commission, 2008).

2.5.6 Air within cleanrooms

The air within cleanrooms is the most likely contamination route, especially in the critical processing zone. This is why air samples in Grade A are the most critical. Other areas of concern include doors and air locks (The United States Pharmacopeial Convention, 2012/a). Furthermore, Agalloco (2005) points out that samples can be just as easily contaminated during the sampling process or testing of the samples.

However, any detected contamination detected during sterility testing, EM testing, product bioburden testing, and process water testing is primarily assumed to be from its source and not a secondary contamination from the sampling act or sample handling in the laboratory. As a consequence, isolators used in the laboratories for microbiological testing, especially for sterility tests but also for EM, bioburden and water testing, have found increased acceptance in the pharmaceutical industry to reduce the probability of secondary contamination during testing (Agalloco, 2005).

Smoke studies are a powerful tool to visualise the air flow within the cleanroom especially in Grade A. Since the most probable contamination route in the APA is airborne, it is apparent that EM samples should be placed near exposed sterile materials. Moreover, areas of concern are also around doors, due to air turbulence, and entry points for the bringing in of materials and equipment. Since the elimination of turbulence within the APA cannot be avoided, it should be minimised by design and control of material flow and personnel movement (The United States Pharmacopeial Convention, 2012/a). Another useful method of identifying contamination risks that is recommended by the United States Pharmacopeial Convention (2012/a) are risk assessment models. In this regard, the application of GIS can help in developing such a risk assessment model through the incorporation of different data layers representing risks categories (e.g. personnel movement, material flow etc.) and combining them to determine the high risk areas within the APA but also in cleanrooms of lower grades.

2.6 Environmental monitoring

2.6.1 Environmental monitoring as quality assurance tool

Pharmaceutical companies with established cleanroom operations have the statutory obligation to demonstrate controlled cleanroom performance using EM of physical parameters, non-viable particles and viable particles (microorganisms also called bioburden) in the air, on surfaces, on personnel, in the products, in auxiliary substances added to the product and in process waters of different purity (European Commission, 2005; ISO 13408-1, 2008; Tierney et al., 2010). The focus of this research is in the area of bioburden recovered in the physical environment (EM), as GIS analysis provides the biggest benefit in this regard.

A suitable facility EM programme should detect atypical changes and trends in the required cleanroom parameters that can compromise the facility's environment and it should facilitate the restoration of normal operating conditions to qualified levels before exceeding established microbial action levels. Furthermore, EM should promptly identify routes of contamination for corrective actions before any product contamination

occurs (Concept Heidelberg, 2004; ISO 14644-5, 2004). In this regard, EM data reflect a state of control of the facility and does not imply sterility of the products manufactured within the cleanrooms (Sutton, 2010). Farrington, as cited in Sutton (2010) expanded on this premise by stating that the relationship of the EM data with the finished product quality is an unproven but commonly held belief. The major issue with EM data is their imprecision. Nevertheless, the EM data trending does help in ascertaining a state of control when assessed with scientific rigour and judgement (Sutton, 2010). ISO 13408-1 (2008) states that EM data shall be evaluated and appropriate actions shall be taken based on the identified risks. The risk assessment for cleanroom manufacturing must be performed over a significant time period for which the contamination recovery rate metric should be ascertained on the basis of an actual findings review within the facility. Another objective of EM besides tracking the ongoing performance, is to refine the microbiological control programme for continuous improvement of the processes. This means that cleanroom performance in regard to physical parameters and microbial recovery rates should become fairly stable within a normal range of variability, and even improve over time when optimum operational conditions are achieved within the facility (The United States Pharmacopeial Convention, 2012/a).

2.6.2 Cleanroom and process validation using environmental monitoring

There are a number of approved tools for achieving biocontamination control thorough risk assessments according to ISO 14698-1 (2003) including Fault Tree Analysis (FTA), Hazard Analysis and Critical Control Point (HACCP) and Failure Mode and Effects Analysis (FMEA), but also any other validated risk assessment system. Any selected risk assessment system must address the following principles:

- Identification of potential hazards to the process or product including probability of occurrence and determination of preventative measures or control
- Designation of risk zones and determination of procedures, operational steps and environmental conditions for each risk zone that can be controlled to eliminate hazards or reduce the probability of occurrence
- Establishment of control limits
- Establishment of scheduled EM
- Establishment of Corrective And Preventive Actions (CAPA) when EM indicates that procedures, operational steps and environmental conditions are not under control
- Establishment of tests and procedures to verify the effectiveness of the biocontamination control system
- Establishment of training procedures
- Establishment and maintenance of appropriate documentation

(ISO 14698-1, 2003).

The commissioning of a cleanroom requires initial room validation using particle, air and surface sampling as well as monitoring of the physical parameters (humidity, temperature, non-viable particles, differential pressure, air exchange rates and air flow velocity) of the cleanroom to validate that the cleanroom can be continuously operated within defined microbiological and physical parameters (The United States Pharmacopeial Convention, 2012/a). The room validation is performed at-rest and in-operation by monitoring these microbiological and physical parameters. The environmental monitoring results from the at-rest demonstrate the general cleanroom performance and the effectiveness of the cleanroom cleaning & disinfection, while the in-operation results demonstrate that the activities within the cleanroom are performed in a controlled and low particle-generating manner and that the cleanroom systems (e.g. air exchange, laminar air flow, etc.) are capable of cleansing the cleanroom air from particles below specified alert and action levels. The validation requires the development of a documented sampling plan for the cleanroom, based on the risk assessment. A documented sampling plan is elemental for assessing and understanding biocontamination data. Sampling should be performed when the area is in-operation during periods of greatest action, e.g. during the highest activity or towards the end of the manufacturing/filling process. In addition, sampling sites must be chosen to reflect and assess the impact of personnel movement and activities within the cleanroom, especially in the APA (ISO 14698-1, 2003).
The sampling plan should consider the cleanliness level of the area and degree of biocontamination control necessary to ensure appropriate aseptic performance of the process (ISO 14698-1, 2003). The basic requirements to consider for a sampling plan based on the risk assessment are:

- Sampling locations
    - Sampling locations must be in proximity of the containers, closures, exposed product and product contact surfaces
    - The sampling at the chosen sampling locations should be able to capture any viable and non-viable particles emitted by the movement and positioning of personnel within the cleanroom
    - The sampling at the chosen sampling locations should be able to capture any viable and non-viable particles emitted due to interventions by personnel in Grade A
      (The United States Pharmacopeial Convention, 2012/a)
- Sampling frequency
- Number of samples

- Sampling method, e.g. quantitative vs. qualitative
- Volume or area to be sampled
- Factors that affect culturing results
- The requirement of neutralisers, rinse fluids, diluents to neutralise cleaners and disinfectants and to remove agar residues (from contact plate monitoring)
- Impact of personnel, equipment, and operations that contribute to biocontamination of the cleanroom
  (ISO 14698-1, 2003)

ISO 14644-2 (2000) recommends the use of a grid approach to disperse sampling locations within the cleanroom as well as to determine the total particulate air quality for cleanroom grade classification. The United States Pharmacopeia (USP) states that this approach can also be used for microbiological sampling points. Grids can add value in determining risk from microbial contamination. However, the USP also states this would require the incorporation of personnel activity to provide meaningful results. The EM risk assessment for the sampling sites selection should take into consideration the impact of personnel movement and tasks carried out within the cleanroom; especially interventions and manipulations within the critical processing zone (The United States Pharmacopeial Convention, 2012/a).

EM encompasses the sampling of non-viable and viable (microorganisms) particles within the cleanrooms, especially in Grade A. Viable monitoring is the detection and enumeration of bacteria, moulds and yeasts which includes monitoring of air, surfaces and personnel using microbiological methods (Tierney et al., 2010). The final product filled in the final containers is also tested using sterility tests (Concept Heidelberg, 2004). Sampling methods are highly variable and many factors can affect microbial recovery and survival rates. Sample-to-sample variation can be extensive and there is limited data for the accuracy of EM methods used in the aseptic processing. For example, surface monitoring with contact plates has been shown to yield microbial recovery rates of <50% even with high inocula. On top of that, recovery rates may be lower in actual cleanroom environments where microorganisms are stressed to varying degrees (The United States Pharmacopeial Convention, 2012/a).

Viable and non-viable particle counts vary within a cleanroom grade and depend on the activities being conducted. The continuous monitoring of non-viable particles using electronic instrumentation does not supply useful information on the microbial count of the air in cleanrooms. However, microorganisms are often attached to particles of 10-20µm in size. Microbiological monitoring cannot identify and quantify all microbes within

a cleanroom. It is therefore a semi-quantitative operation that gives a snapshot of the environmental conditions within a cleanroom. It is a fact that a lack of microbial recovery does not automatically infer sterility; it only means that the number of microorganisms at a given point is below the detection level of the analytical system since the sensitivity of any microbial sampling system is not known and subsequently is often estimated. Therefore, there should be no false sense of security if microbial recovery rates within the cleanroom are low (The United States Pharmacopeial Convention, 2012/a).

It is well documented that microbial counts can vary by 10-30% depending upon the choice of growth medium used and incubation conditions chosen. The use of molecular methods in recent times has shown that the microorganisms present in any environment are far more diverse and they far outnumber those which grow on commonly used media. There is a diverse group of oligophilic microorganisms (able to grow at an extremely low concentration of nutrients) that do not grow on conventional media and it has been demonstrated that a substantial portion of these microorganisms can be cultured by using a dilute and diverse nutrient medium (Nagarkar, Ravetkar, & Watve, 2001). However, to capture most microorganisms would require the use and validation of a wide range of media, which would be impractical for routine monitoring. Therefore, sterility assurance is best achieved by focusing on and reducing personnel and other contamination factors through facility and process design. The greatest risk reduction to the product can be attained by reducing and, if possible, eliminating personnel intervention in Grade A through process design (The United States Pharmacopeial Convention, 2012/a).

EM can be performed in-operation, which is the state where the manufacturing process is in-operation with personnel working in the cleanrooms, or at-rest which is the state where the manufacturing process is operable or operating but no personnel are present (European Commission, 2008). EM in the APA of new cleanrooms must be initially executed as-built (with no production equipment, materials or personnel) and at-rest. At-rest monitoring must also be performed after shutdowns and before commencing manufacturing. After initial set up and validation, routine EM in the APA is executed in-operation (ISO 14698-1, 2003). Established cleaning and disinfection procedures in cleanrooms are evaluated for their effectiveness by at-rest EM. All disinfectants have to be validated for their effectiveness on the in-house microbial isolate collection. Furthermore, microbiological challenge tests can be performed in case microorganisms associated with negative trends are assumed to be less sensitive to certain applied disinfectants. (Concept Heidelberg, 2004; European Commission, 2008).

The final EM validation report contains a summary of the results and any deviations from established alert- and action levels observed and corrective and preventive actions taken as well as any influence on the validation and the room status. Any recommended changes for the cleanroom or EM programme (such as EM methods or sampling locations) should also be stated in the report (European Commission, 2001). Furthermore, the validation report may also include a risk assessment for the microbiological sampling locations that are used for the routine EM if more locations have been selected in the validation sampling plan to encompass unknown process variables. The established EM programme is then incorporated in SOPs including a list of the sampling locations, sample size, frequency and sampling equipment to be used for consistent and reproducible sampling (Concept Heidelberg, 2004; ISO 13408-1, 2008). EM sampling plans should also be regularly reviewed and adjusted based on the risk analysis and contamination rates (The United States Pharmacopeial Convention, 2012/a).

2.6.3 Environmental monitoring methods

There are a number of EM sampling methods to assess and control the microbiological status of cleanrooms. Currently, nearly all of these methods require the growth and recovery of microorganisms that may be damaged because of environmental stresses (e.g. from disinfectants) and hence difficult to detect and retrieve (The United States Pharmacopeial Convention, 2012/a). Air and surfaces of cleanrooms are monitored in regular intervals using specially designed agar plates to detect microorganisms at specific high risk and/or microbial exposure locations which are determined through the risk assessment during the initial cleanroom validation and revalidation programme. EM sampling methods and procedures are selected and carried out in accordance to applicable EM SOPs that reflect instructions provided by the device manufacturer (e.g. a microbiological air sampling device). Microbiological air samples are collected in-operation during manufacturing/filling, while surface samples are taken after the production/filling of the product to prevent product contamination during sampling. However, surface samples taken right after production/filling are still considered in-operation. (Health Canada, 2009; ISO 13408-1, 2008; The United States Pharmacopeial Convention, 2012/b; Tierney et al., 2010).

Growth media used for EM but also for product and water bioburden testing must be sterile and demonstrate the recovery of a wide range of microorganism (bacteria, yeasts and moulds) via growth promoting assessments, using the microbiological in-house isolates as well as a selection of microorganisms from an approved culture collection, e.g. American Type Culture Collection (ATCC), for every new batch of

media. The standard culture medium used for EM must not be selective and should contain additives to neutralise residual antimicrobial chemicals present at the sampling locations. In spite of this, selective media can be useful for the detection of specific microorganisms, including objectionable organisms that are difficult to recover with standard nutrient media, and which should be absent in non-sterile products or in intermediates of sterile products during manufacture before sterilisation filtration. The detection of moulds and yeasts can be enhanced through additional EM monitoring, using Sabourraud Dextrose Agar (SDA), for example, as a selective medium. However, this selective monitoring of specific microorganisms must be performed in addition to the conventional EM monitoring using standard culture media that promote the growth of a large variety of microorganisms, such as Tryptic Soy Agar (TSA) (ISO 13408-1, 2008; The United States Pharmacopeial Convention, 2012/b; Tierney et al., 2010). The packaging of culture media brought into the cleanroom has to be decontaminated through disinfection and removal of packaging layers (double or triple plastic wrapping, often gamma sterilised with agar plates) (ISO 14698-1, 2003).

The air of cleanrooms is monitored through active extraction using a Microbiological Air Sampling (MAS) device at a set volume for a certain time over the sterile agar medium (active air monitoring). In addition, filter plates can be placed in the MAS-device to filter a set volume of air after which they are aseptically laid onto an agar plate for incubation (ISO 13408-1, 2008; ISO 14698-1, 2003; The United States Pharmacopeial Convention, 2012/a; Tierney et al., 2010). Passive air monitoring is performed as a semi-quantitative or qualitative method, especially in Grade A, with the greatest risk of product contamination using agar plates that are exposed to the laminar air flow, thereby capturing any settling viable organisms. The results of passive air monitoring can be interpreted as cfu/4h (or cfu/plate) depending on the validated air exposure time of the agar (Concept Heidelberg, 2004; ISO 14698-1, 2003). The EM programme must take into consideration such factors as the expected concentration of viable particles and the ability to detect low level of microorganisms (ISO 13408-1, 2008; ISO 14698-1, 2003). This is why different sampling volumes are set for viable air monitoring in different cleanroom grades (Reich et al., 2003), such as 1000L in Grade A/A2/B, 500L in Grade C and 200L in Grade D, to obtain an appropriate number of cfu (e.g. ≤250 cfu/agar plate) on the agar plates for accurate counting.

Surface monitoring is performed with either contact agar plates (see Figure 6 on page 35), using the same agar types as for air monitoring, or swabs, which both capture the microorganisms through contact and adhesion (Concept Heidelberg, 2004; Tierney et al., 2010). In addition, particles on surfaces can be detected using the tape lift method and other surface particle detection methods (ISO 14644-5, 2004). Surface monitoring is performed on a regular basis on such surfaces as floors, walls, ceilings and equipment, especially with product contact. While it is apparent that microorganisms in the air can contaminate the product during aseptic processing or filling operations, especially in Grade A, it is difficult to establish with confidence if any microorganisms recovered from surfaces that do not have direct product contact are endangering the product (The United States Pharmacopeial Convention, 2012/a). Personnel working in the APA must be monitored on their gloved fingertips using contact plates at the minimum daily or per shift. Moreover, samples from the gowns (chest, forearms and hood) must also be taken at defined intervals. The recovered microorganisms from all EM samples must be identified and the overall monitoring data are assessed for negative trends as well as to evaluate required actions such as personnel retraining (Health Canada, 2009; ISO 13408-1, 2008).

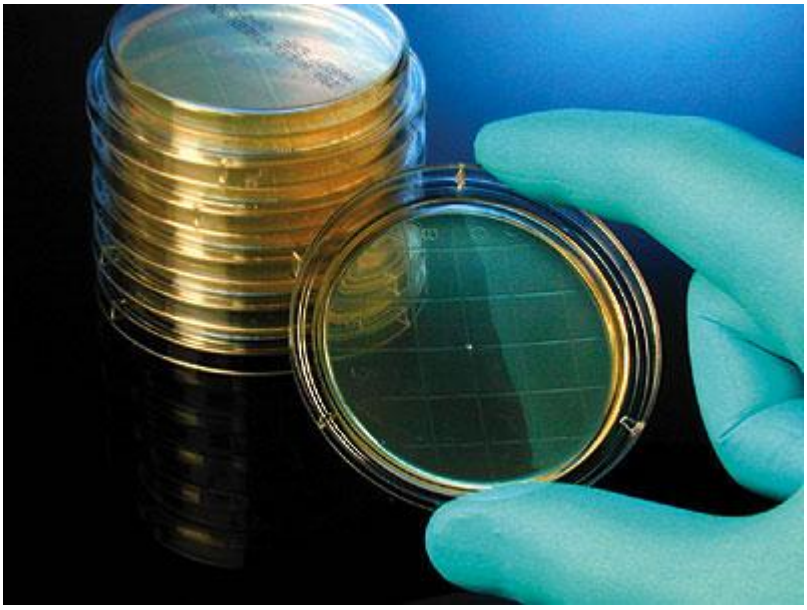

Figure 6: Contact agar plates (Cole-Parmer, 2013)

The EM samples are incubated according to their validated temperatures, such as 20-25°C in the course of 5-7 days for SDA (for yeasts and moulds), or 30-35°C in the course of 2-3 days for total aerobic microbial count, as recommended by Tierney et al. (2010). On the contrary, ISO 14698-1 (2003) recommends an incubation period of 2-5 days for bacteria, while Reich et al. (2003) recommends 3-4 days. Cundell (2004) advocates for the most conservative approach by recommending an incubation period

of 7 days at 35°C followed by another 7 days at 25°C. However, this would hinder a root cause analysis due to the long duration between sample collection and results. For this reason, the ideal incubation temperature, duration, and conditions should enable the growth of the types of microorganisms expected, and must therefore be validated. This is also recommended by the United States Pharmacopeial Convention (2012/a). In addition, specific atmospheric conditions and incubation times may be necessary when micro-aerophilic, thermophilic, anaerobic, fastidious, or nutritionally deficient microorganisms are expected in the cleanrooms. The agar plates are then analysed by counting the colony forming units per volume (e.g. 1000L for Grade A) or per plate (25cm²) for air and surface monitoring respectively. The incubated agar plates can be examined at appropriate intervals in the course of the incubation period to obtain preliminary results if necessary (e.g. after shutdown of cleanrooms) (Concept Heidelberg, 2004; ISO 14698-1, 2003; Tierney et al., 2010).

Pharmaceutical companies have the responsibility of maintaining and keeping records which is part of Good Manufacturing Practices (GMP) and Good Documenting Practices (GDP) (Simmons, 2010). The microbiological data can be stored as a hard copy, but in most pharmaceutical companies nowadays electronic data are stored with a validated program e.g. a Structured Query Language Laboratory Information Management System (SQL*LIMS), for further analysis. Correct data input and output into computer programs or related systems must be verified for accuracy by validation to which the degree depends on the complexity and importance of the data. Furthermore, backup storage and archiving of important process data is required by GMP (Concept Heidelberg, 2009).

2.7 Assessment of recovered microorganisms, trending and result interpretation

2.7.1 Analysis of monitoring data

Once the EM samples including the microbiological samples have been taken according to the EM sampling plan, and the examination of samples has provided enumerated results, these results have to be matched with established alert and action levels (ISO 14698-2, 2003). Microbiological alert and action levels are determined and set appropriate to the cleanroom grade and zone classifications, fields of application and to what is achievable using current technology. Alert and action levels are either based on regulatory requirements or are initially determined from the data trend analysis using statistical methods (Cundell, 2004). As part of the microbiological data trending, a review of the EM results should be performed periodically to verify the continuing effectiveness of EM and analytical methods. Part of this review should be a calculation and reassessment of the current alert and action levels for continuous

improvement or significant changes in the microbiological status of the different cleanroom grades. Any microbiological process deviation such as an action level excursion or significant negative trend must be investigated. Each out-of-specification result (deviation) requires an evaluation to determine if it was a true result. Any single uncorrelated contamination event may be a false positive, as sampling itself necessitates an aseptic intervention in conventional cleanrooms. Therefore, the detected microbiological action level excursions and significant negative trends should include an investigation and exclusion of the possibility of a sampling error, preparation error or testing error in the laboratory (ISO 14698-2, 2003; The United States Pharmacopeial Convention, 2012/a).

Certain microorganisms in non-sterile products and intermediate products have the ability to reduce or inactivate therapeutic activity through biochemical changes to the product. Furthermore, microbial toxins (such as exotoxins, endotoxins, and mycotoxins) and other pyrogens generated by the microorganisms in the product are a risk to the patient who receives the drugs. Therefore, two reasons for the identification of microorganisms in any sample (product, environment, water, etc.), but especially in the APA, is the detection of “objectionable microorganisms”, which are unwanted in the intermediate or final product, and groups of microorganisms such as Gram-negative rods (which can be objectionable organisms) that can add to the endotoxin content of the final product if present in sufficient numbers in the process water, excipients, or intermediate product itself (Cundell, 2004). The term “objectionable organism” is stated in § 211.113(a) of the 21 Code of Federal Regulations (CFR) 210/211, as cited in Concept Heidelberg (2009), which states that written procedures are required to prevent objectionable microorganisms in drug products not required to be sterile. However, this would not apply to the intermediate production steps of sterile products and it would not require looking for objectionable microorganisms within cleanrooms, but this was demanded from pharmaceutical companies during audits by the Food and Drug Administration (FDA) through legislative means, and can therefore be judged as state-of-the-art or cGMP (Sutton, 2010).

Maximum acceptable levels and the absence of certain objectionable microorganisms are defined for non-sterile products in Section <1111> of the United States Pharmacopeia (The United States Pharmacopeial Convention, 2012/c), in Appendix XVI of the British Pharmacopoeia (British Pharmacopoeia Commission, 2012) and in section 5.1.4 of the European Pharmacopoeia (European Directorate for the Quality of Medicines & HealthCare, 2011/b), categorised by the route of administration and therefore risk to the patient (e.g. nasal vs. cutaneous).The levels are set from 200-2000

cfu/ml or cfu/g for total aerobic microbial counts and 20-200 cfu/ml or cfu/g for total combined yeast and mould counts. Some microorganisms must, however, be absent in the drugs, including *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Candida albicans*, and bile-tolerant Gram-negative bacteria. Furthermore, this list of microorganisms is not exclusive and it may be necessary to evaluate and include other microorganisms depending on the potential to reduce or inactivate the therapeutic activity of the drug, the manufacturing process, and the nature of the product (British Pharmacopoeia Commission, 2012; European Directorate for the Quality of Medicines & HealthCare, 2011/b; The United States Pharmacopeial Convention, 2012/c). Consequently, pharmaceutical companies that manufacture sterile products, such as the one used in this research, define the above stated microorganisms and category as “objectionable organisms in the intermediate products of aseptically filled sterile drugs and also in the cleanroom environment of the APA”, as there are no other specified requirements. However, this seems to be a compromise for the lack of legislative guidance, and Cundell (2004) would not recommend this approach, but does not deliver an alternative. A different approach for determining objectionable organisms could be for example based on a risk assessment considering the microbiological flora within the cleanrooms, microbial species and genera that can be recovered with the chosen media and incubation conditions (i.e. no anaerobic microorganisms if no anaerobic incubation), and microorganisms that must not be in the cleanrooms (distinguished by grades, e.g. no moulds in the APA).

The result of each individual EM sample within the cleanroom is compared to the set alert and action levels, with any non-conformance to those levels seen as a deviation to the validated routine manufacturing conditions. Any deviation thereof triggers an investigation into the root-cause of the non-conformance and requires corrective and preventive actions. The number and type of microorganisms must be considered for the assessment of the deviation’s impact on product quality (ISO 13408-1, 2008). Investigations should include a review of physical and operational parameters in the cleanroom (humidity, temperature etc.), training status of operators, occurrence of non-routine events, area maintenance documentation and cleaning/disinfection documentation (The United States Pharmacopeial Convention, 2012/a).

It is imperative to assess correlations between sampling sites, since trends on single sample points or sites may not give a complete picture of the cleanroom environment being monitored. Examples of negative trends that should lead to a root-cause investigation are:

- Excursions above action levels

- Alert level excursions indicating a potential negative trend
- Increased occurrence of microbial counts, above historical levels, but below the action level
- Occurrences of unusual events, e.g. extended mechanical breakdown
- Internal or external audit observations indicating a potential increase in contamination risk
- Repeated occurrences of microorganisms not often detected
- High numbers of microorganisms at a single sampling point

(ISO 13408-1, 2008)

The first step in primary screening and microbial characterisation of obtained isolates through EM is the differentiation by colony morphology and subsequent Gram staining of individual bacterial colonies, along with microscopy for cell morphology. Gram staining is a very important step, especially with further biochemical identification, as a false Gram staining result that provides the basis for further biochemical identification tests will lead to a false pathway of identification and will most likely yield a wrong result. This is why Gram-negative and Gram-positive controls should be routinely performed to detect staining errors. Furthermore, older cultures may yield Gram-variable results and this is why the USP recommends that individual colonies should be streaked onto fresh media before Gram-staining and further identification (The United States Pharmacopeial Convention, 2012/e). The level of microbial identification will depend on the type of sample and clean room grade, and whether the root-cause investigation requires further identification. Broad categories based on colony morphology, cell morphology (e.g. rods, cocci and spore forming microorganisms), Gram-staining (Gram-positive and Gram-negative), and certain diagnostic biochemical reactions (catalase, oxidase and coagulase activity) to categorise the recovered microorganism are adequate for grades C and D, for example, if no action level excursion occurred. Identification of at least to the genus level has to be established for action level excursions and any isolates from the APA using validated laboratory procedures, (e.g. biochemical identification, immunological identification, fatty acid profiles, mass spectrometry, or genotypic methods using nucleic acid amplification techniques). The identification can help to determine the contamination source, appropriate actions and the determination of appropriate cleaning and disinfecting procedures, e.g. sporicidal disinfection (European Directorate for the Quality of Medicines & HealthCare, 2011/a; ISO 14698-1, 2003; The United States Pharmacopeial Convention, 2012/e).

The identification and characterisation of recovered microorganisms offers valuable information for the EM programme, and identified environmental isolates may sometimes correlate with other isolates detected in the cleanroom or product. In addition, EM should also be performed in laboratories to aid an investigation into a possible contamination of microbiological samples within the laboratory environment (Concept Heidelberg, 2004; ISO 13408-1, 2008; The United States Pharmacopeial Convention, 2012/a). Furthermore, in some cases, EM data trending can reveal microbial migration into the APA from Grade D. Therefore, Gram-staining alone in lower grades such as C and D may not help in detecting trends and entry of microorganisms into the APA, which may endanger the product during aseptic filling (Sutton, 2010).

The differentiation by diagnostic biochemical reactions or any other validated identification method will yield a broad microbiological differentiation database, including the number and variety of obtained objectionable organisms. Table 3 on page 41 shows the primary screening and objectionable organisms in the APA of this study.

Table 3: Preliminary screening and objectionable organisms in the APA

Colony morphology and Gram-staining	**Diagnostic pre-biochemical testing**	**Objectionable organism genus**	**Objectionable organism species**
Moulds	n.a.	all	all
Yeasts	n.a.	all	all
Gram-positive cocci	Agglutination test	*Staphylococcus*	*S. aureus*
		Streptococcus	all
		Enterococcus	all
Gram-negative cocci	n.a.	all	all
Gram-positive rods without spores	Specialised agar media	*Listeria*	*spp.*
		Corynebacterium	*diphteriae*
Gram-positive rods with spores	Anaerobe agar media & incubation/spore staining	*Bacillus*	*anthracis*
Gram-negative rods	Oxidase positive	*Bordatella*	*spp.*
	Anaerobe agar media and incubation	*Pasteurella*	*spp.*
		Flavobacterium	*meningosepticum*
		Burkholderia	*pseudomallei*
	Oxidase positive and lactose positive	*Escherichia*	all
		Ekinella	
		Klebsiella	
		Proteus	
		Citrobacter	
		Salmonella	
		Shigella	
		Edwardsiella	
		Hafnia	
		Enterobacter	
		Yersinia	
		Serratia	
		Pantoea	
		Vibrio	

2.7.2 Microbiological isolates in cleanrooms

The cleanroom is generally thought to be an inhospitable environment that should not support many microorganisms for prolonged periods (La Duc et al., 2007). Having knowledge of the microbial flora within the cleanroom environment aids in a detection of unusual microorganisms and high microbial numbers of a certain type (Concept Heidelberg, 2004). Furthermore, information of the microbial flora within the cleanrooms aids in contamination control, fosters quality assurance and is part of cGMP (Sandle, 2011). Significant changes in the microbial flora must be assessed in the data review. The results from the data analysis/review should be escalated to the responsible managers on a regular basis (e.g. through a management review), and should include trends and the status of investigations (Concept Heidelberg, 2004). Sandle argues that a controlled cleanroom environment should have a relatively static microbial flora with little diversity, as any change may indicate new cleanroom contamination routes from unusual sources, or the formation of resistant strains. In spite of the importance of knowing the microbial flora within pharmaceutical cleanrooms, there have been very few studies published in recent years in this regard. Yet only through these studies can microbiologists benchmark the types and frequency recovery of the microorganisms within pharmaceutical cleanrooms. In essence, the examination of the microbial flora allows microbiologists to compare their own microbial cleanroom flora data against that collected by similar organisations operating cleanrooms (Sandle, 2011).

The most frequently isolated microorganisms in the pharmaceutical cleanroom environment according to Cundell (2004) in ascending order are:

- Human skin bacteria including *M. luteus*, *M. varians*, *S. epidermidis*, *S. simulans*, *S. hominis*, of the Gram-positive cocci group;
- *Corynebacteria spp.* of the Gram-positive rods without endospores group;
- Airborne bacterial spores including *B. thuringiensis*, *B. subtilis*, *B. sphaericus*, *B. cereus*;
- Infrequently Gram-negative bacteria including *Burkholderia cepacia*, *Enterobacter cloacae*, etc. and
- Occasionally airborne fungal spores including *Aspergillus niger*, *Penicillium spp.*, etc.

Gram-positive cocci and to some extend Gram-positive rods without endospores are found on human skin in high numbers and are readily shed. These are best controlled through proper personnel gowning procedures and aseptic techniques. Bacterial endospores are commonly found in dust and cellulosic material, and are often dispersed through material traffic (Cundell, 2004). A consistent recovery of Gram-

positive spore forming microorganisms on successive occasions or within short time durations may indicate inadequate cleaning practices (Sandle, 2011). Fungal spores, on the other hand are released by actively growing fungal colonies on or within damp substrates, including vegetation surrounding the manufacturing plant, and cardboard packaging brought into air locks (Cundell, 2004). Gram-negative bacteria can be found in cleanroom areas with water sources, and low numbers can be related to personnel hygiene issues, such as coughing and sneezing (Sandle, 2011).

A study conducted by Sandle (2011) reviewed the types, trends and patterns of over 9000 microbial isolates from a range of different cleanroom grades (grades A, B, C and D) in pharmaceutical companies in the UK over a period of 9 years (2001-2009). The study showed that the most commonly isolated microorganisms within cleanrooms are Gram-positive bacteria (97%) and that the vast majority of bacteria isolated from cleanrooms are aerobic or facultatively aerobic mesophilic bacteria. 81% of microorganism recovered in the APA (grades A and B) were Gram-positive cocci, 13% were Gram-positive spore forming rods, 3% were Gram-positive non-spore forming rods, 2% were Gram-negative bacteria and 1% were fungi. The ratio of these microorganisms over the 9 year period remained relatively constant. The majority of genera isolated in the APA can be seen in Table 4 on page 43 which constituted >80% of all isolates. The top 10 of identified microorganism are depicted in Table 5 on page 44. Lyophilisers and the use of specialised gases (e.g. nitrogen) are sometimes related to microorganism that can survive in anaerobic conditions, while the recovery of thermophiles from cleanrooms is very infrequent. The most frequently isolated genera of fungi within cleanrooms include *Penicillium*, *Aspergillus* and *Trychophyton*.

Table 4: Majority of genera isolated in the APA in the study by (Sandle, 2011)

Genus	**Grades A and B**	**Grades C and D**
	Percent (number)	
Micrococcus (and related)	38% (2571)	40%
Staphylococcus	21% (1397)	11%
Bacillus (and related)	13% (875)	10%
Corynebacterium (and related)	3% (198)	5%
Rhodococcus	<1% (35)	Not stated
Pseudomonas (and related)	<1% (30)	8%

Fungi	Not stated	3%

Table 5: Top ten most commonly isolated species in Grade A and B according to the study by Sandle (2011)

Rank number	Species or genus	As a percent of all isolates
1	*Micrococcus luteus*	26%
2	*Micrococcus lylae*	10%
3	*Staphylococcus spp.*	6%
4	*Micrococcus spp.*	5%
5	*Bacillus sphaericus/ fusiformis*	5%
6	*Staphylococcus epidermidis*	4%
7	*Staphylococcus capitis*	2%
8	*Staphylococcus hominis*	2%
9	*Bacillus spp.*	2%
10	*Staphylococcus haemolyticus*	2%

The 2500 isolates from grades C and D cleanrooms showed a pattern of far greater variety compared with the APA. Sandle (2011) concluded that this was due to the higher personnel flow and level of control, especially with higher recoveries of Gram-positive rods being dragged into these areas, but also due to the contained water sources (sinks and water outlets connected to equipment) in grades C and D, which is reflected in higher Gram-negative rods isolates. Furthermore, most items brought into the APA are sterilised while those brought into grades C and D areas are only unpacked and decontaminated using disinfectants. No Gram-negative cocci were recovered in any cleanroom (Sandle, 2011).

A study conducted by La Duc et al. (2007) that focused on the recovery and characterisation of bacteria capable of tolerating the severe and extreme conditions of cleanroom environments (cleanrooms used for spacecraft assembly), which are termed extremophilic or extremotolerant, showed a variety of bacteria that they were able to recover. The study demonstrated that 25% of the cultivatable bacteria were Gram-negative bacteria, while 75% were Gram-positive bacteria. This correlates with the study by Sandle where 78% of isolates in grades C and D could be classified as Gram-positive bacteria. Some of the Gram-negative bacteria in the study of La Duc et al. (2007) included *Pantoea stewartii*, *Acinetobacter baumannii*, *Pseudomonas syringae*, *Sphingomonas dokdonensis*. Some of the Gram-positive *Staphylococci* included *S.*

epidermidis, *S. pasteuri*, *S. cohnii*, and *S. hominis*. Other Gram-positive cocci included *Micrococcus luteus*, *Kocuria marina* and *Rhodococcus fascians*. 40% of the cultivable microbial population isolated were members of the genus *Bacillus* with the most common species being *Bacillus megaterium*, but also *B. cereus*, *B. pumilus*, *B. flexus*, *B. firmus*, and *B. circulans* were among the isolates (La Duc et al., 2007). However, Gram-positive non-spore forming rods were not recovered in this study and spore-forming bacteria were far more scarcely recovered than anticipated since they have the ability to form resistant endospores that are capable of withstanding a variety of severe environmental conditions including many disinfectants. Although the cleanroom was not used for pharmaceutical manufacturing, the study shows the type and variety of bacteria that can be found in a cleanroom environment, and that many different and difficult to culture microorganisms (i.e. extremotolerant) can be recovered if different culture media and recovery techniques are used. This finding was also proven by Nagarkar et al (2001).

2.7.3 Microbiological data trending

The quality control unit of a pharmaceutical company must check long-term and near-term trends (e.g. quarterly, monthly, weekly, daily) in personnel and environmental monitoring data. Trend reports should include data generated by operator, room, shift location, or other parameters. Furthermore, specialised data reports containing, e.g., a particular isolate over an annual period should be established to investigate obtained data beyond established alert and action levels for appropriate actions (Concept Heidelberg, 2004). Before statistical calculations with the obtained data can be carried out, it is necessary to compress the data for better understanding. This can be done in a quantitative way, using descriptive statistics, or a qualitative way, by grouping results to form frequency charts and tables. The quintessence of any statistical tool is the extrapolation from the sample to the microbial population within the cleanroom, although the sample may not represent the microbial population within the sampled cleanroom area at large. This risk can be enumerated and decreased to a tolerable level by using statistics and probability sampling. Data from single samples are often not significant and microbiological monitoring tools may have a high degree of variability. Therefore, graphic depiction of the data collected over a set time period using statistical control charting aids in ascertaining sampling variation from trends, and changes in the data, and determines if the results are still within the specification limits (ISO 14698-2, 2003).

Separate trending categories should be applied such as filling line, personnel and the different products manufactured in order to more accurately monitor potential

contamination sources. Unusual events such as atypically high microbial counts or maintenance work in cleanrooms should be reviewed and assessed for impact on product sterility (Concept Heidelberg, 2004). However, the trending of microbiological data represents a challenge as there is inherent count variability due to sample variation, assay variation, and population variation, which is especially apparent in the APA where low action levels are established and microbial counts are low. In addition, microbiological sampling in Grade A will reveal no microbial counts at all most of the time. Therefore, a cluster of 5 samples with 1 cfu may well have more implication over a single sample with 5 cfu (Hussong & Madsen, 2004). Recovery and growth in microbiological assays have a standard variability in a range of ±0.5 log10 (The United States Pharmacopeial Convention, 2012/a). Scientific studies on active microbiological air sampling using MAS-devices indicate a 10-fold variability. Consequently, numerical values within a 10-fold range are, from an analytically aspect, not significantly different, and microbiologists should use practical scientific judgement when dealing with action level excursions. Therefore, mean contamination recovery rates should be ascertained for each cleanroom environment and changes in contamination recovery rates within a cleanroom or a given site may indicate the need for corrective and preventive actions (The United States Pharmacopeial Convention, 2012/a). In this regard, spatial analysis tools such as hot spot analysis could be useful to determine hot spots with high contamination recovery rates that may be influenced by adjacent cleanrooms.

Nonetheless, Cundell (2004) states that the pharmaceutical industry has undergone an unfortunate trend driven to take on recommended target microbial levels for cleanrooms and use them for the evaluation of microbial monitoring data in order to find negative trends in the microbial performance of the cleanrooms. The methods used for microbiological EM, such as contact plates, swabs, and active and passive air sampling have low precision, poor recoveries, and microbial numbers that are normally at or close to the detection limit of the methods. For example, it is difficult to defend that 1 cfu/contact plate on the surface in Grade A (Grade A action level:>1 cfu/plate) would be acceptable, while 3 cfu/plate would trigger a non-conformance deviation given the heterogeneity of the microbial distribution within the cleanroom and the analytical capabilities of the method (Cundell, 2004).

This is why the United States Pharmacopeial Convention (2012/a) recommends to trend contamination recovery rates rather than the number of colonies recovered from a given sample, owing to the variability of the microbiological sampling methods. The United States Pharmacopeia (USP) endorses a recovery rate in percent instead of the cfu number in the cleanroom environment. The recovery rate is a percentage of the

number of samples with microbial contaminants compared with samples without microbial growth. The detection frequency should be trended on a monthly basis. Unfortunately, the USP does not recommend a recovery rate for Grade D (ISO 9 – in-operation). An investigation and the implementation of appropriate corrective and preventive actions are required if the set action levels, which are based on process capabilities, are exceeded or if detection frequencies (recovery rates) exceed the recommendations from the USP in Table 6 on page 47. A key consideration of unusually high numbers of colonies detected within the cleanroom - especially in the APA - is whether it is an isolated incident or unusual pattern, and possibly a systemic failure that can be correlated with other incidences. The data should also be reviewed retrospectively to determine any unusual and potentially correlated patterns in recovery rates that could represent a systemic failure in the quality system. Moreover, the USP states that any excursion resulting in >15 cfu in Grade A requires a thorough investigation, and must trigger a non-conformance report and CAPA initiation. Examples of corrective actions to negative trends according to the USP are:

- Increased surveillance and constructive feedback on personnel practices
- Revision of the cleaning/disinfection programme, including the selection, application method, and frequency of disinfection
- A review of microbial sampling methods
- Additional training on correct gowning procedures

(The United States Pharmacopeial Convention, 2012/a)

Table 6: Recommended contamination recovery rate limits in USP section <1116>

Room classification	**Active air sampling (%)**	**Settle plates with max. 4h exposure (%)**	**Surface contact plate or swab monitoring (%)**	**Personnel monitoring of gloves and garment (%)**
ISO 5 (Grade A)	<1	<1	<1	<1
ISO 6 (Grade A/B)	<3	<3	<3	<3
ISO 7 (Grade B)	<5	<5	<5	<5
ISO 8 (Grade C)	<10	<10	<10	<10

(The United States Pharmacopeial Convention, 2012/a)

Any data trending, whether for cleanroom grade barrier breaches, product bioburden issues, or process water bioburden issues should always take into consideration a short- and long-term EM trend analysis. In some cases, EM data trending can reveal

migration of microorganisms into the APA from either controlled Grade C and D areas or even uncontrolled areas (e.g. NCA, GA.). Therefore, establishing an acceptable programme for differentiating microorganisms in grades C and D can often be instrumental in detecting such trends. The programme should at the minimum require genus/species level identification of isolates from these ancillary areas at frequent intervals to:

- Establish an updated current database of microorganisms present in the facility during processing
- Demonstrate that cleaning and sanitization/disinfection procedures are effective

(Concept Heidelberg, 2004; ISO 13408-1, 2008; The United States Pharmacopeial Convention, 2012/a).

The evaluation, interpretation and significance of biocontamination data must take the following factors into account:

- Systems to process the collected results e.g. correlation analysis, statistical analysis, artificial intelligence
- Grouping of results to focus on important deviations and trends e.g. data stratification
- Method used to express the results (e.g. quantitatively, qualitatively, or graphically) and the units of measurements used
- Potential problems and robustness of the analytical methods
- Control charting
- Trend analysis
- Interpretation, estimation and reporting of results
- Constitution of viable particle spectrum and its variability over time
- The relationships between indirect and direct testing, and
- Results obtained from different sampling sites within the APA and the rest of the cleanroom

  (ISO 14698-2, 2003).

Consistent EM methods will yield a microbial database for sound data comparison, interpretation and trending (Reich et al., 2003). The data trending is part of Statistical Process Control (SPC) with the aim of monitoring manufacturing processes to detect changes in process performance (Korakianiti & Rekkas, 2011). The microbial database must be updated with new microbial isolates that are difficult to identify, or which are not in the database but were recovered during monitoring. For this, the microbial identification tools and methods should be appropriate technology, selected for accuracy to use in routine microbiological identification testing (The United States

Pharmacopeial Convention, 2012/e). A big benefit of a microbial database lies in the comparison of recovered microorganisms from microbiological process deviations (in EM, process water bioburden and product bioburden) or even just unusual results within the microbial dataset. Furthermore, non-sterile products or unsuccessful media-fills detected during sterility testing of the final container or the bulk product can be compared with the microbial data to find the fault in the quality assurance system, by determining if the specific microbial species found in the non-sterile product or medium has been recovered elsewhere in the facility, and how it might have been introduced into the product/container, hence the root cause. In this regard, serological or genotypic methods used for the identification of isolated microorganisms are especially valuable for microbiological process deviation investigations or non-sterile products, and these have been shown to be more specific and accurate than traditional phenotypic and biochemical methods (Concept Heidelberg, 2004; ISO 14698-1, 2003; The United States Pharmacopeial Convention, 2012/e).

There are different genotypic methods based on molecular identification that can be used to determine if microbial species are of the same strain and most likely are from the same origin (cell) such as riboprinting, pulsed-field gel electrophoresis, optical mapping, whole genome ordered restriction and arbitrarily primed polymerase chain reaction (The United States Pharmacopeial Convention, 2012/a). One of the molecular-based genotypic methods, based on arbitrarily primed polymerase chain reaction that can be useful to determine a clonal relationship between microbial isolates is the Randomly Amplified Polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR) technique. This technique is a type of PCR reaction where the DNA segments are amplified at random and for which relatively few unique DNA sequences are compared. The RAPD-PCR method can differentiate between genetically distinct individuals. It is especially useful for microorganisms that have not had the attention of the scientific community e.g. commensal human skin bacteria often isolated in cleanrooms (Kumar & Gurusubramanian, 2011). The RAPD-PCR can ascertain if for example microbial isolates of the same species found in the manufacturing environment and in the product are clonally related and thus have originated from the same source i.e. the same cell (Eneroth, Ahrne, & Molin, 2000). Moreover, this technique has also been successfully utilised in epidemiological studies (Wong, Linton, Jalal, & Millar, 1994).

In spite of technological advances (i.e. molecular techniques in microbial identification and comparison), Hussong & Madsen (2004) state that it is almost impossible to determine the root cause of a single microbiological action level excursion. They further conclude that meaningful conclusions can only be drawn when microbiological data are

related to time or location of multiple microbiological observations. In this regard, GIS may be the right tool for spatial analysis of multiple microbiological observations and reflects the conclusion of Hussong & Madsen (2004) which states that process improvement requires the search for new process analytical technologies for environmental microbiology.

2.8 Research problem and questions

In summary, there is a range of requirements, including available methods, covering the EM cleanroom data collection, analysis, and data interpretation aimed at quality assurance in pharmaceutical manufacturing. Although conventional cleanroom EM data trending using statistical analysis (e.g. control charting) can provide valuable information on negative and positive trends in the data, it does not link the data to the geographic environment. Therefore, vital information on the microbiological status of the cleanroom may be left out. This research will use the ArcGIS 10.1 software to spatially analyse the EM data provided by the pharmaceutical company to answer the following questions:

a) Which areas in the manufacturing cleanroom facility have higher air and surface microbial counts?
b) Have the air and surface microbial counts changed over time based on 3 years of data?
c) Is there a certain microbiological flora associated with certain areas of the facility?
d) What is/are the potential contamination route(s) in the cleanrooms?

3 Materials and Methods

3.1 Cleanroom study area

This study used GIS to spatially analyse the microbiological environmental monitoring data obtained from a pharmaceutical company, and generated in the cleanroom environment of the pharmaceutical manufacturing facility. The cleanroom study area consists of the ground floor of a pharmaceutical facility that contains four APA sterile filling suites (with grades A, A2 and B) connected by an A2 corridor with lyophilizers. The APA is surrounded by cleanroom support areas (Grade D) and Grade A Air Supply (GAAS) areas for vial crimping. There are rooms with no cleanroom validated status called General Area (GA), which already have certain gowning procedures and specified cleaning intervals, but no EM, that lead to cleanroom areas of Grade D. In addition, there are Non Classified Areas (NCAs) that include other parts of the building such as staircases, technical areas etc.

A2 cleanroom areas in this study incorporate areas for the aseptic transfer of the products in their final containers, where the product is partially sealed with lyophilisation stoppers, and an aseptic packaging area in sterile filling suite 4, where the inner packaging of the already sealed final container (syringe) must be sterile. For a detailed view of the facility including cleanroom grades, see Figure 7 on page 52. The only cleanroom grade lacking on this floor is Grade C, which is used for the formulation and sterile filtration of the products on another floor. The products are then inline transferred to the sterile filling suites after Cleaning in Place (CIP) and Sterilisation in Place (SIP) of the lines. The pharmaceutical company is located in the EU, but the exact location and coordinates are not stated within this thesis, due to the sensitivity of the data and potential breach of a prior contractual arrangement with the pharmaceutical company that provided the data.

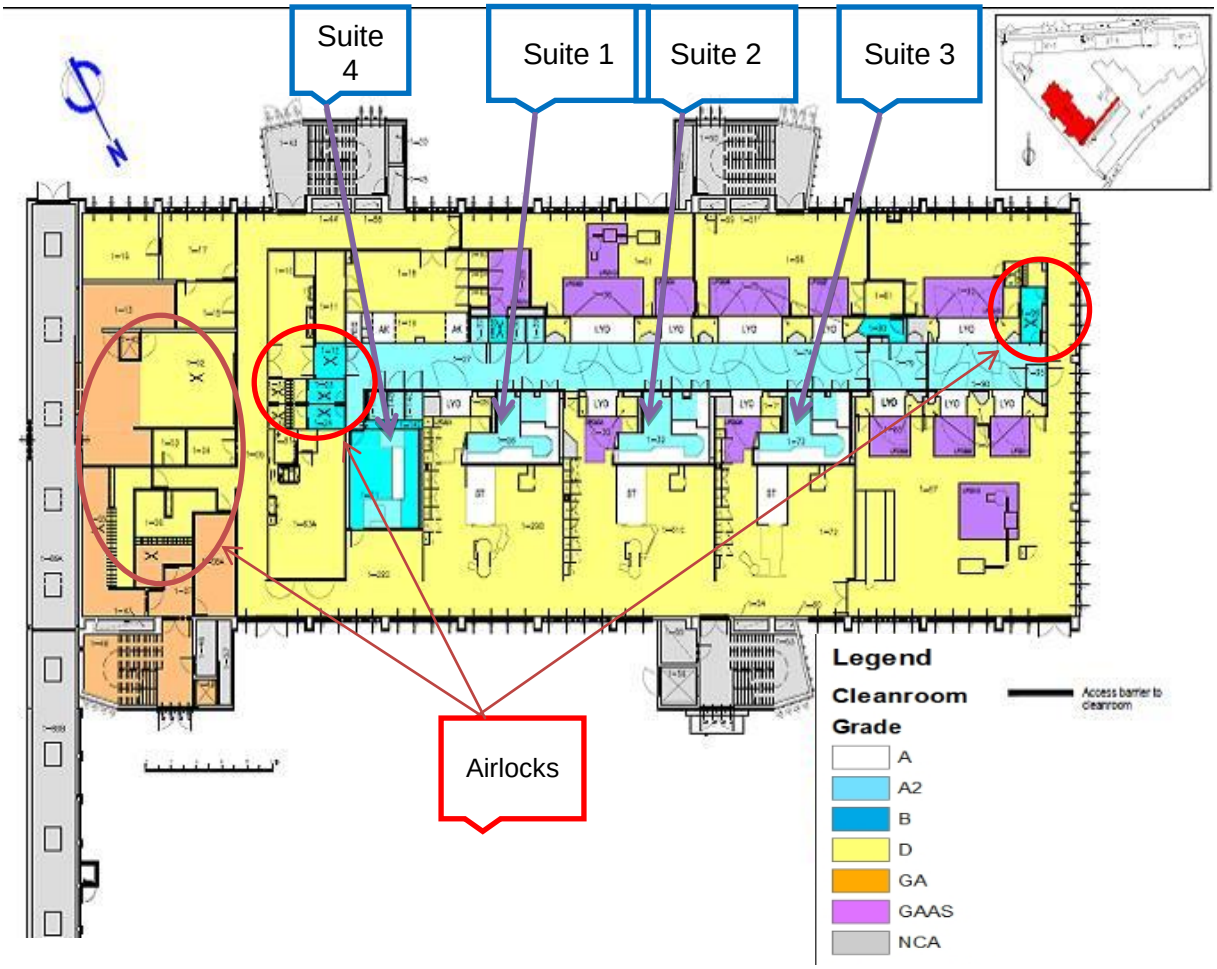

Figure 7: CAD drawing of the study facility including overview of cleanroom grades, filling suites and air locks

The study used ArcGIS 10.1 software by Esri that provides spatial analysis tools which can be used to compile, manage and analyse geographic information (Esri Inc., n.d.). ArcGIS is an extensive software package with many extensions. It is widely used by many professionals and was therefore chosen for this research. The geographic data used in this study came in the form of CAD drawings of the pharmaceutical manufacturing facility's ground floor, containing air sampling locations, as well as the different cleanroom grades, filling suites, air locks and support areas (see Figure 7 on page 52) etc. The CAD drawings were transformed into polygon (cleanrooms) and point (air sampling points) shapefiles, which were spatially adjusted and displaced onto the raster layer of the manufacturing facility's location. For a detailed view of the GIS study process to obtain the polygon shapefile of the manufacturing facility see the cartographic model in
Figure 8 on page 53.

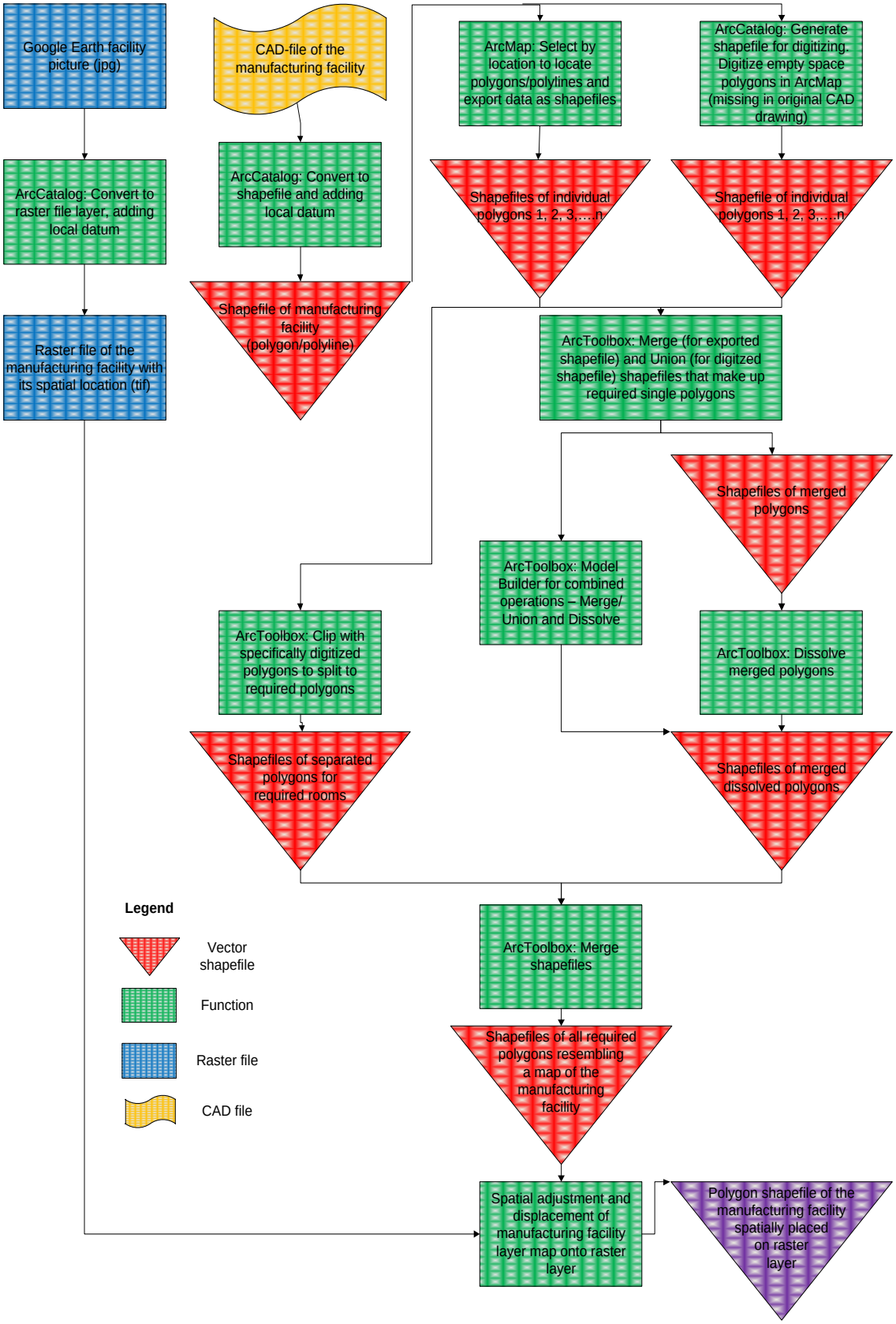

Figure 8: Cartographic model of the process used to obtain the polygon shapefile of the manufacturing facility

The attribute data used for this research has been extracted from the company's Structured Query Language Laboratory Information Management System (SQL*LIMS) into an Excel file. The attribute data are microbiological data generated over three years (2009-2011) of operational air and surface environmental monitoring in the manufacturing facility's cleanrooms of grades A, A2, B and D. For an overview of the EM viable particle monitoring frequency regime, refer to Table 7 on page 54.

Table 7: Viable particle monitoring frequencies within cleanroom grades

Grade	**Viable air monitoring**		**Surface monitoring**		
				Personnel	
	Active air monitoring	**Settle plates**	**Room and equipment**	**Gloves and sleeves**	**Garment**
A	once per lot filling	once per lot filling	daily	following critical interventions	not required
A2	once per lot filling	once per lot filling	daily	once per lot filling and every time before leaving the APA	
B	once per lot filling	not required	daily	once per lot filling and every time after leaving the APA	
C	weekly	not required	weekly	weekly	
D	monthly	not required	monthly	not required	

The study included only viable air and surface monitoring data, ignoring viable monitoring data of personnel working in the clean rooms, as it is very difficult to track which (and when) rooms have been entered by personnel (especially in Grade D) and hence which microbial count data should be attributed to which clean room. In addition, the microbiological data of mobile equipment such as a cleanroom fork lift and cleanroom trolleys were also not included, as the movement of these items is also difficult to track. Grade A Air Supply (GAAS) microbial count data were not available for this research, as the GAAS environment was not in place during the period of the data collection. The Grade A air supply unit in the lower right corner of the CAD-drawing has also been removed, as this area used to be classified as Grade D. Furthermore, no at-rest microbiological data have been considered for this research, as the microbial counts are relatively low and at-rest monitoring is only performed within these cleanrooms after shut-downs (generally once a year), or to check the cleaning status; hence, the data quantity is not sufficient for analysis.

3.2 Data preparation

Before the data could be incorporated into the attribute table of the various layers, it had to be transformed. The distribution of microbiological data is essentially asymmetrical and most of the time does not reflect a normal distribution. It therefore requires an appropriate data transformation, such as a log transformation (Moore & McCabe, 2006) or a reverse hyperbolic sine transformation (Zhang, Fortney, Tilford, & Rost, 2000), depending on the data distribution. The microbiological data in this study, on the other hand, were transformed to percentage recovery rates, as this is recommended in section <1116> of the United States Pharmacopeia (The United States Pharmacopeial Convention, 2012/a) and hence can be viewed as a kind of regulatory statistics tool of choice for microbiological data analysis by the US Food and Drug Administration (FDA). The microbial percentage Recovery Rates (RRs) of the microbial count attribute data (surface and air) for each cleanroom were calculated in Excel. The formula to calculate the microbial percentage recovery rate is $\sum \frac{the\ number\ of\ values > 0\ cfu}{(sum\ of\ 0\ cfu + > 0 cfu\ values)} * 100$. For a summary of the data set with the air and surface RRs total (2009/2010/2011) and per annum including raw data (sum of 0 values vs. sum of >0 values per room), see Appendix 1: Microbiological recovery rate data summary on page 126.

3.3 Methodology for Question 1: Which areas in the manufacturing cleanroom facility have higher air and surface microbial counts?

After completing the process of generating the polygon shapefile of the study facility and placing it onto its geographic location, a point shapefile was generated showing the air sampling points per cleanroom (one point per cleanroom or grade) within the study facility. The previously calculated microbiological surface and air monitoring percentage recovery rates were then incorporated from the Excel file into the attribute tables of the surface and air shapefile layers. From this, choropleth maps were generated showing the microbial percentage recovery rates per cleanroom (surface monitoring) and per air sampling point (air monitoring) respectively. The polygon shapefile layer with the microbial surface monitoring percentage recovery rates was then joined to the point shapefile layer of the manufacturing facility containing the air percentage recovery rates. The air and surface microbial percentage recovery rates were then added together in the attribute table using the field calculator in the attribute table. The data from the APA (grades A, A2 and B) were combined and analysed separately to the Grade D data, as microbial percentage recovery rates cannot be compared between these cleanroom grades due to different environmental background conditions, such as lower HEPA filter rates and significantly higher allowable viable and non-viable particle rates in Grade D compared with the APA.

The Hot Spot Analysis - Getis-Ord Gi* tool, a spatial statistics tool was then deployed to find statistically significant (p-value: <0.05) hot spot (and cold spot) cleanroom areas (the APA and Grade D were again separated). Choropleth maps of the manufacturing facility were then generated showing the results of the hot spot analysis as well as the air and surface microbiological recovery rates. For a detailed view of the GIS study process, see the Cartographic model in Figure 9 on page 57 to obtain the choropleth maps of the manufacturing facility cleanrooms with the results of the hot spot analysis.

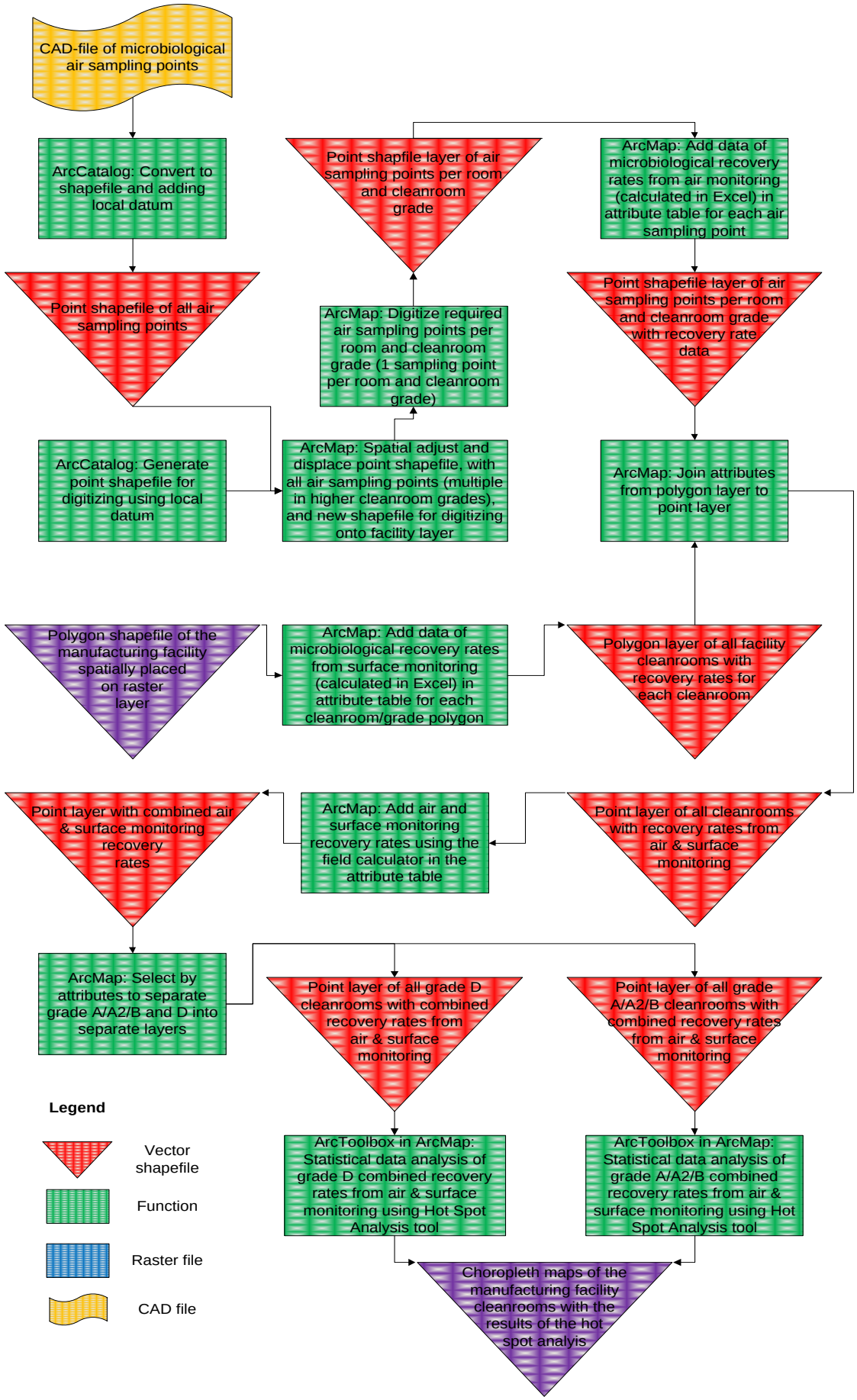

Figure 9: Cartographic model of the process used to obtain the choropleth maps of the manufacturing facility cleanrooms with the results of the hot spot analysis

3.3.1 Hot Spot Analysis (Getis-Ord Gi*)

A distribution of attributes or features within a defined area will produce a pattern. The geographic pattern can be completely dispersed or completely clustered. A pattern that falls in between the two extremes is random. Determining patterns in geographic data is useful to better understand the data and act accordingly. A hot spot analysis based on a Z-score calculation can be used if features create a clustered pattern (Mitchell, 2005). Z-scores show how many standard deviations the observations are away from the mean and in which direction (Moore & McCabe, 2006). The calculation formula for the hot spot analysis can be seen in Figure 10 on page 58.

The hot spot analysis would identify the location of these clusters of attributes and/or features at a local scale by a measure and by their statistical significance. The local G-statistic shows where clusters of high or low values are located spatially by comparing neighbouring features within a set distance. The statistic indicates the degree to which each feature is surrounded by similar high or low values using, for example colour coded mapping. Obtained high Gi* values reflect statistically significant clusters of high and low values - hot and cold spots (Mitchell, 2005). A high Gi* value with a z-score above 1.96 suggests that high values (i.e. recovery rates) tend to be near each other (hot spot), while a low Gi* value with a z-score below -1.96 suggests that low values tend to be near each other (cold spot) (Wang, 2006).

The Getis-Ord local statistic is given as:

$$G_i^* = \frac{\sum_{j=1}^{n} w_{i,j}x_j - \bar{X}\sum_{j=1}^{n} w_{i,j}}{S\sqrt{\frac{\left[n\sum_{j=1}^{n} w_{i,j}^2 - \left(\sum_{j=1}^{n} w_{i,j}\right)^2\right]}{n-1}}} \quad (1)$$

where x_j is the attribute value for feature j, $w_{i,j}$ is the spatial weight between feature i and j, n is equal to the total number of features and:

$$\bar{X} = \frac{\sum_{j=1}^{n} x_j}{n} \quad (2)$$

$$S = \sqrt{\frac{\sum_{j=1}^{n} x_j^2}{n} - \left(\bar{X}\right)^2} \quad (3)$$

The G_i^* statistic is a z-score so no further calculations are required.

Figure 10: Calculation formula for the Hot Spot Analysis – Getis-Ord Gi* (ArcGIS 10.1, 2012)

The following factors influence the results of Gi* (best practice guidelines to avoid distorted results):

- Small numbers of features (<30) may alter the results due to outliers
- Results can be skewed due to features which have fewer neighbours, near the edge of the study area
- Global pattern clusters are less obvious than local patterns
  (Mitchell, 2005).
- All features must have at least one neighbour
- No feature shall have all other features as neighbours
- Each feature should have about eight neighbours each in case the input values are skewed
  (Esri Inc., 2010)
- Geographic features which are close to each other are prone to be more similar than distant features; this phenomenon is referred to as "spatial autocorrelation". This violates the presumption that observations are independent (Mitchell, 2005).

These best practice guidelines to avoid distorted results have been taken into consideration during the analysis and the Discussion section of this report.

3.4 Methodology for Question 2: Have the air and surface microbial counts changed over time based on 3 years of data?

The surface and air microbial count recovery rates for each cleanroom were calculated in Excel for each year (2009, 2010 and 2011). The results were then incorporated in separate columns of the facility shapefile attribute table and depicted in three choropleth maps (one for every year) showing any change in recovery rates (up or down) throughout the three year period. The number of 0 values and the number of >0 values for each cleanroom/grade were then processed in the Excel software using the chi-square (χ^2) statistics to determine if any changes in 0 vs. >0 values over the three year period are statistically significant. The χ^2 statistics has the formula $\sum \frac{(observed-expected)^2}{expected}$ (Moore & McCabe, 2006) where the observed counts are the number of 0 values and >0 values for each cleanroom/grade for each year in a 3 x 2 table [(years in columns) x (0/>0 values in rows)]. The expected values are obtained using the formula $\sum \frac{row\ total * column\ total}{n}$.

The null hypothesis (H0) is that the number of 0 values vs. the number of >0 values in each cleanroom class have not significantly changed over the three year period (the data spread is due to chance) while the alternative hypothesis (Ha) is that they have

changed significantly. The p-value of the χ^2 statistic has to be <5% to reject the null hypothesis, otherwise the observed values deviate from the expected due to chance alone. A new column was generated in which a number code (0-4) was added for the obtained results (e.g. 1 = lower RR, 2 = higher RR etc.) and new choropleth maps were generated showing the results of the analysis including the statistically significant change in air and surface RR data. The results of the analysis were used to determine if the microbiological data are stable, or if the recovery rates have even decreased (continuous improvement), which is a central statement in section <1116> of the United States Pharmacopoeia (The United States Pharmacopeial Convention, 2012/a).

3.5 Methodology for Question 3: Is there a certain microbiological flora associated to certain areas of the facility?

The data for the microbiological flora from the cleanroom study area (air and surface as well as personnel) were extracted from the company's SQL*LIMS database via a Chrystal Report query using the SAP Business Objects software. The timeframe chosen for the query was from the year 2009-2011. The data includes in-operation EM (i.e. routine, cleanroom validation and special investigation). The microbial data from Grade D and from the APA were categorised separately in the following major classes stated in Table 8 on page 60.

Table 8: Microbial data categories for Grade D and the APA

Grade D categories	**APA categories**
1. Moulds	1. Moulds (objectionable organism in the APA)
2. Yeasts	2. Yeasts (objectionable organism in the APA)
3. Gram-positive cocci, catalase positive (abbreviated G+ve cocci C+)	3. Gram-positive cocci, catalase positive (abbreviated G+ve cocci, C+) 4. Gram-positive cocci, catalase negative (abbreviated G+ve cocci, C-, some of which are objectionable organisms)
4. Gram-positive rods without endospores (abbreviated G+ve rods, S-)	5. Gram-positive rods without endospores (abbreviated G+ve rods, S-)
5. Gram-positive rods with endospores (abbreviated G+ve rods, S+)	6. Gram-positive rods with endospores (abbreviated G+ve rods, S+)

6. Gram-negative rods (abbreviated G-ve rods)	7. Gram-negative rods, oxidase positive (abbreviated G-ve rods, O+) 8. Gram-negative rods, Oxidase negative, Lactose positive (abbreviated G-ve rods, O-, L+, most of which are objectionable organisms)

For a list of the Objectionable organisms (moulds, yeasts and classified bacterial objectionable organisms), see Table 3 on page 41. The microorganisms that are classified as objectionable organisms in the APA of the pharmaceutical company in this research comprise of almost all the require microorganisms as defined by the European, British and United States Pharmacopeias (and more), including those in the bile-tolerant Gram-negative bacteria category, which reflects most organisms in the coliform category (see Table 3 on page 41). However, *Pseudomonas aeruginosa* is not defined as an objectionable organism in the APA, but should be in this category, as this microbial species is an objectionable organism in Water for Injection, used for pharmaceutical manufacturing, and in intermediate pharmaceuticals. The rationale for the classification of Grade D microbiological data is that there are no objectionable organisms in Grade D, and the identification to genus or species level is only executed if an action level excursion occurred at a particular sample location as well as for cleanroom validation and special investigation EM (but not routine EM). Consequently, the Grade D microbial data are primarily classified according to colony morphology and Gram-staining.

The rationale for the classification of the APA microbiological data is that there are objectionable organisms defined in the APA and all microorganisms are identified. This is why the additional classes G+ve cocci, C- and G-ve rods, O-, L+ have been introduced into the analysis, as these classes contain objectionable bacterial organisms. One limitation is that microorganisms in the Grade B air locks are only identified if an action level excursion occurred. Furthermore, the air locks of all grades in the study area have only been monitored in-operation since 2010, as they were previously monitored at-rest, thus there is no operational data available before this date. The at-rest microbiological data cannot be compared with the operational data, as the recovery rate is rather low showing merely a functional cleaning regime in the air locks (Concept Heidelberg, 2004).

The data from the Chrystal report file was incorporated into an excel table and the percentage microbial class distribution was calculated. Graphs were generated in Excel

for a graphical depiction and easy comparison. The distribution of these microbiological classes within the cleanrooms was analysed to find a pattern in the data and an association of certain microbiological classes to the different cleanroom grades.
The identified microorganisms were then analysed and separated by cleanroom grades according to the microbial categories/classes (e.g. G+ve cocci C+ etc.). The rationale for this separation is that the microbiological class distribution between the different cleanroom grades should be different due to different cleanroom uses. For example, the logistical tasks in Grade D may have a somewhat different microbiological flora than cleanroom grades within the APA. Grade B areas do not have a unidirectional air flow, while Grade A2 areas do. Grade A areas were also separated, as they should have the lowest microbiological recovery rates and perhaps fewer microbiological classes.

The top three identified microorganisms within their microbiological classes, separated by cleanroom grades, were analysed for their potential origin and distribution within the cleanroom areas and grades. Topley & Wilson's Microbiology & Microbial Infections: Bacteriology (Borriello, Murray, & Funke, 2005a, 2005b), Bergey's Manual of Determinative Bacteriology (Krieg & Holt, 1994) and other literature sources were consulted to determine the natural habitat and thus the potential origin of the microorganisms. The results of the microbiological analysis were depicted in the form of a top three microbial species/genus recovery table for each microbial class separated by cleanroom areas. The identified microorganisms within each microbiological class and their distribution within the separated cleanroom grades were then analysed.

3.6 Methodology for Question 4: What is/are the potential contamination route(s) in the cleanrooms?

The previously generated layers with the hot spot analysis and air & surface microbial recovery rates were combined; the areas that had the highest microbial recovery rates (air and surface) including the areas with statistically significant hot spots were selected by attributes and exported as a separate point layer. A layer with line segments depicting the movement of material and personnel was digitised onto the previously generated facility cleanroom layer to determine the contamination routes in Grade D, and subsequently, into the APA. For a detailed view of the GIS study process to obtain the choropleth map of the manufacturing facility cleanrooms with material & personnel flow, combined high recovery rates and hot spots see Figure 11 on page 64. The determined microbial distribution ascertained in the first three research questions was used, along with the information from the literature review, as a basis from which the

methodology was developed to answer this question. The results of the analysis were compared with the potential contamination routes listed in the literature review to see if there was a correlation. A matrix diagram table was generated for additional cleanroom contamination risks and contamination routes in Grade D and the APA, derived from the literature review. The matrix diagram table is a six sigma quality tool used to organise and methodically display potential root causes and the facts about them (Pyzdek, 2001). Recommendations were made based on the findings of this research and the findings from the literature review which show some cGMP shortcomings and subsequently potential for improvements in the quality assurance systems of this manufacturing facility.

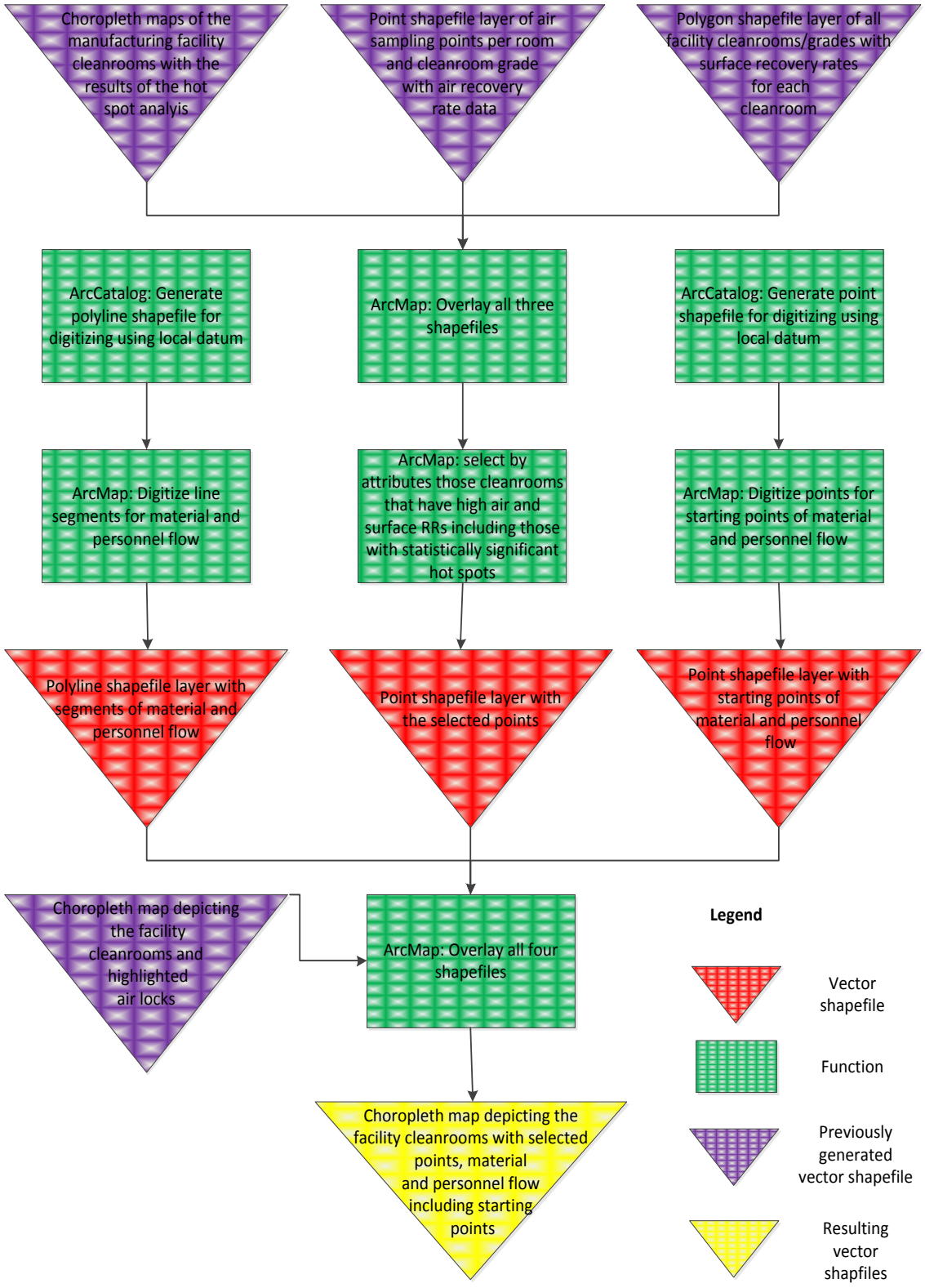

Figure 11: Cartographic model of the process used to obtain the choropleth map of the facility cleanrooms with material & personnel flow, combined high recovery rates and hot spots

4 Findings

The output of the CAD drawing conversion into a polygon shapefile of the pharmaceutical manufacturing facility's ground floor containing the cleanrooms spatially adjusted and displaced onto the raster layer of the manufacturing facility's location can be seen in Figure 12 on page 65.

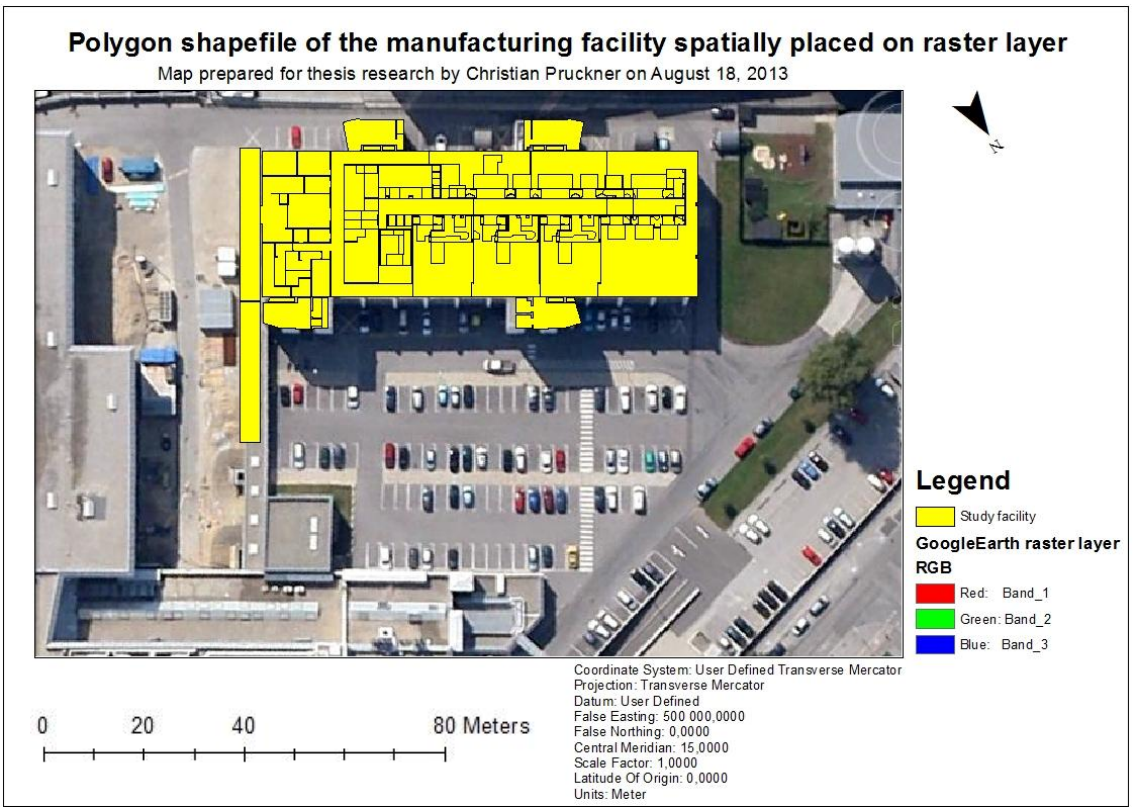

Figure 12: Polygon shapefile of the manufacturing facility spatially placed on raster layer

Figure 13 on page 66 shows a map (shapefile) of the facility with all cleanroom grades and other areas on the ground floor of the building. Personnel enter the Grade D personnel air locks from the GA through the one chamber men's air lock (room No. 6) and women's air lock (room No. 5), while the material flow into Grade D is through the one chamber material air lock (room No. 2). The air locks are highlighted as shaded areas in Figure 13. The GA to Grade D air locks (material and personnel) are one-chamber air locks with the GA on one side and Grade D on the other (where EM is performed) separated by a red line on the floor. Each staff member is trained in the correct aseptic entering of Grade D through these air locks as a standardised procedure, with hand washing, disinfection, gowning, etc. The white and blue areas (grades A/A2/B) in the centre of the ground floor constitute the APA which is connected by the two chamber personnel air locks in the north-east (rooms No. 33/23 - women's air lock and 34/24 - men's air lock) and in the north-west (rooms No. 97/96 – unisex air lock) of the building, leading to the long Grade A2 corridor (rooms No. 27/74/79/90).

The personnel air locks between Grade D and the APA are double chamber air locks where the first chamber is classes as Grade D and the second chamber as Grade B. The colours for each cleanroom grade reflect the colour spectrum standard used for pharmaceutical cleanroom CAD drawings, and the north arrow serves as an aid for orientation.

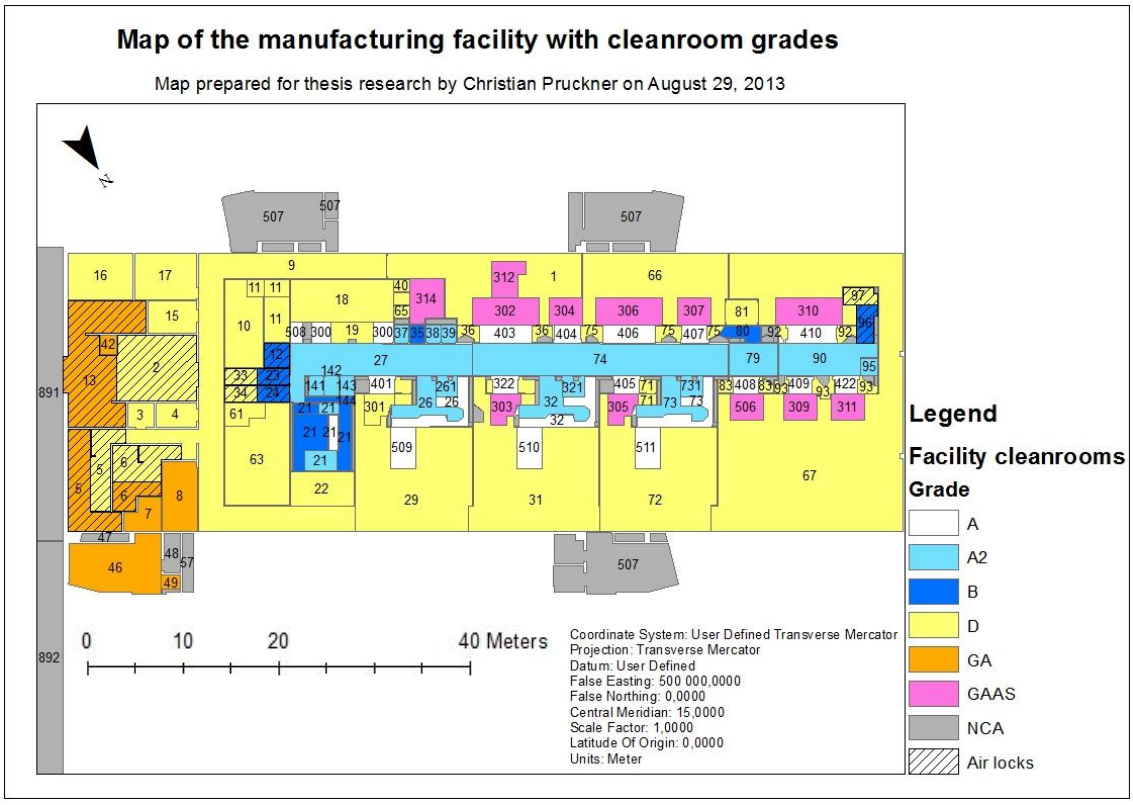

Figure 13: Map of the manufacturing facility with cleanroom grades

4.1 Which areas in the manufacturing cleanroom facility have higher air and surface microbial counts?

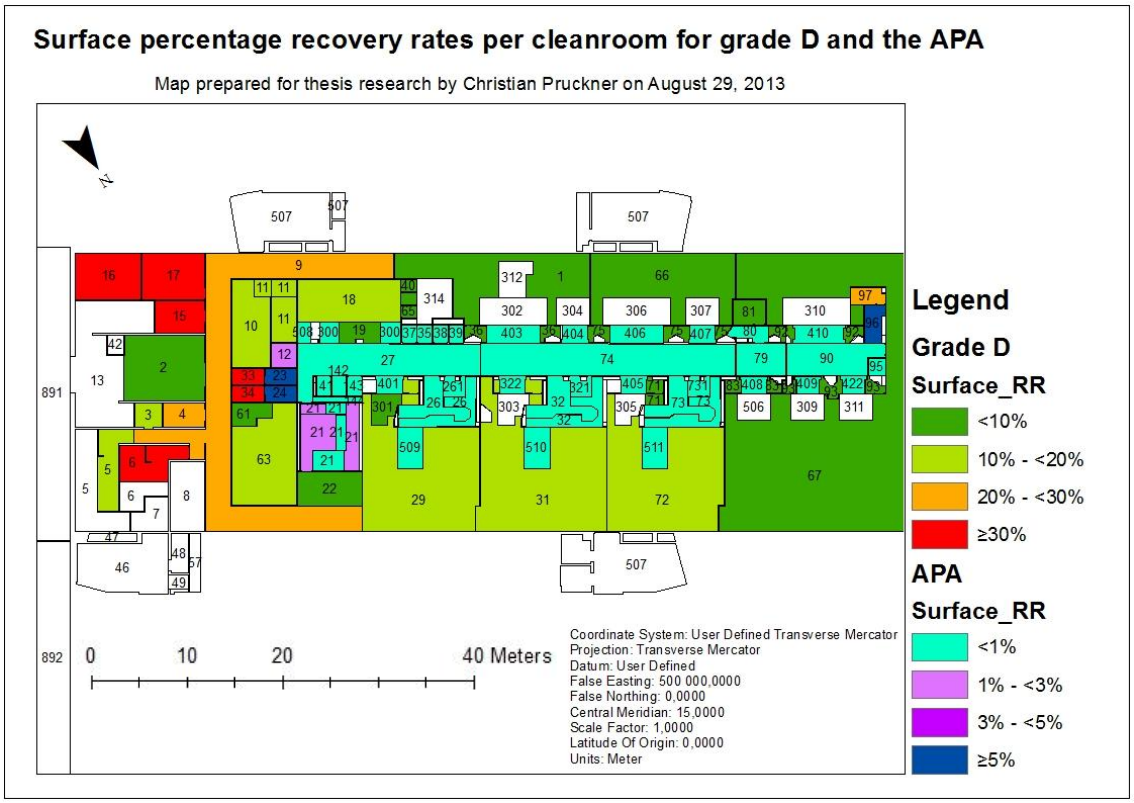

Figure 14: Surface percentage recovery rates per cleanroom for Grade D and the APA

Figure 14 on page 67 shows the obtained choropleth map of the study facility with the results of the calculated microbial surface percentage Recovery Rates (RR) per cleanroom. The classifications (cut-off points) for the RR in the choropleth map were chosen according to the USP section <1116> recovery rate guidelines (Grade A: <1%, ISO 6: <3%, Grade B: <5%, Grade C: <10%) (The United States Pharmacopeial Convention, 2012/a), see Table 6 on page 47. ISO 6 has a slightly lower non-viable particle threshold than Grade A2 and can therefore be viewed as more stringent than Grade A2. Since there is no recovery rate limit recommended by the USP for Grade D, a classification of <10%, 10% - <20%, 20% - <30%, and ≥30% was set based on the distribution of the microbiological surface monitoring data. In addition, if the Grade D microbial action level were to be taken as a basis, then a recovery rate for Grade D could be around 25%, as the microbial action level in Grade D is around 2.5 times higher than in Grade C (2.5*10%).

The highest microbiological surface recovery rates in Grade D (≥40%) are in the documenting rooms for personnel (rooms 16, 17, and 15). High microbial surface recovery rates are also in the Grade D personnel air locks (rooms No. 6, 33, 34, and 97). The first chamber of the Grade D men's air lock (room No. 34) that leads to the

APA has a higher surface recovery rate of 40.41% than the Grade D women's air lock (room No. 33 - 30.11%), or the Grade D unisex air lock (room No. 97 - 29.62%) that also lead to the APA. This is unexpected, as there are fewer men working in the APA than women (coarse ratio of 1:2). Furthermore, rooms No. 4 (documenting room) and No. 9 (Grade D corridor) have also higher surface recovery rates (20% - <30%). The north-west side of the facility has lower surface recovery rates, and interestingly room No. 2 (material air lock) has a low surface recovery rate as well, despite being an air lock.

The surface recovery rates in the APA are generally quite low. The only exceptions are the women's air lock (room No. 23 – 6.34%), men's air lock (room No. 24 – 20.96%), and unisex air lock (room No. 96 – 10.65%). The highest surface recovery rate of 20.96% in the Grade B men's air lock (room No. 24) reflects the high surface recovery rate in the adjacent Grade D men's air lock. The single chamber Grade B material air lock in the APA (room No. 12) has a lower surface recovery rate (2.27%) when compared with the Grade B personnel air locks. What is also noteworthy is that Grade B in sterile filling suite 4 (room No. 21 – 1.63%) has a higher recovery rate compared with the other cleanrooms in the APA. Overall, with the exception of the personnel air locks, the obtained surface recovery rates are all below the recommended limits of USP section <1116>. However, there is no recommended air and surface recovery rate limit for Grade A2 in the USP section <1116>. If we assume a limit of around <2%, which is rounded down half way between the <1% for Grade A and <5% for Grade B, then this would be also true for Grade A2.

Figure 15 on page 69 shows that the air microbial percentage recovery rates in Grade D look quite different to their surface recovery rate counterpart. The percentage recovery rates from air monitoring are much higher with ≥30% in most of the Grade D rooms. The highest recovery rate, however, can be found in the Grade D corridor (room No. 9) with 82.84%. The vial washing rooms for sterile filling suites 1-3 (rooms 29, 31 and 72 – 64.07%, 64.81% and 57.78% respectively) have also high air recovery rates. The other Grade D areas that have higher recovery rates are the equipment washing room (room No. 10 – 35.71%) and the equipment preparation room (room No. 18 – 46.83%). Furthermore, the documenting rooms (rooms No. 17, 16 and 15 – 47.62%, 46.03% and 42.86% respectively) have also higher air recovery rates. On the other hand, the air recovery rates of the Grade D air locks (rooms No. 2, 5, 6, 33, 34 and 97) are not significantly higher compared with the other Grade D cleanrooms that have ≥30% recovery rates.

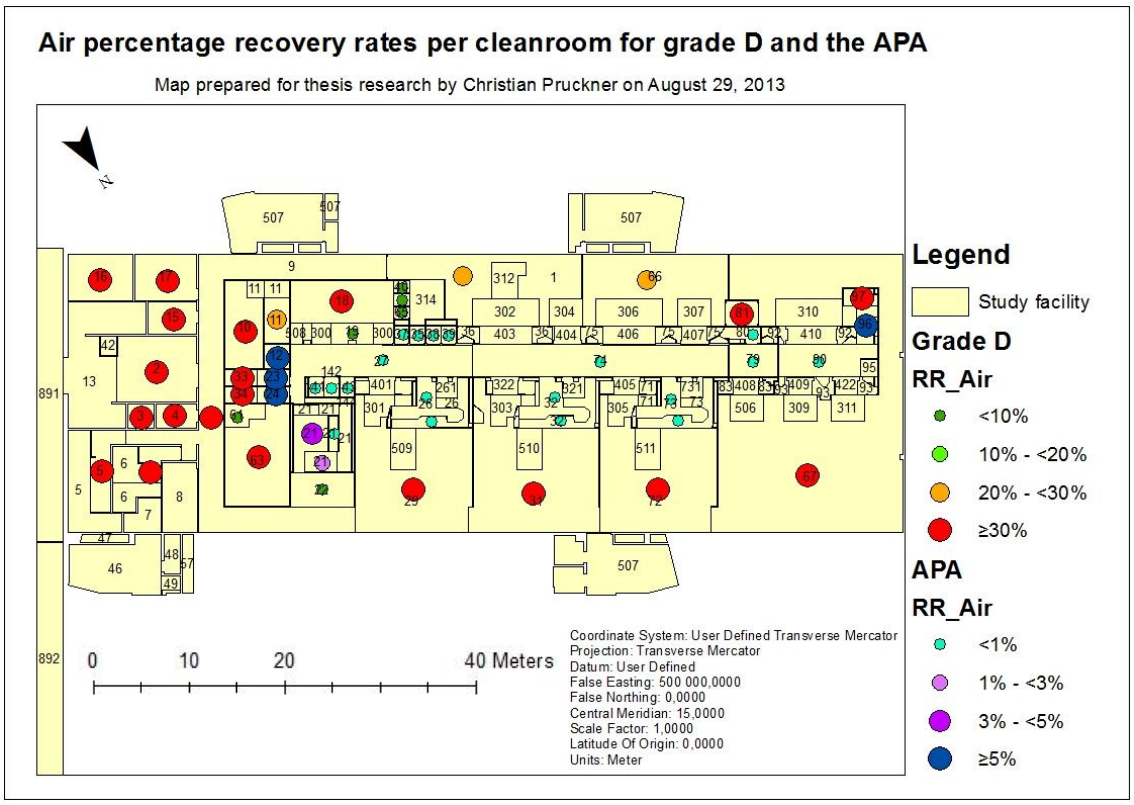

Figure 15: Air percentage recovery rates per cleanroom for Grade D and the APA

The highest recovery rates in the APA are in the men's, women's, unisex, and material air locks with ≥5% (rooms No. 12, 23, 24 and 96 – 9.98%, 16.67%, 23.30% and 11.15% respectively) which in essence reflect the higher surface recovery rates. Grade B in sterile filling suite 4 (room No. 21) has also a higher recovery rate of 4.28%. The obtained values for the Grade B air locks are above the recommended <5% recovery rate for Grade B by USP section <1116> (The United States Pharmacopeial

Convention, 2012/a). Furthermore, Grade A2 in sterile filling suite 4 (room 21) has also a higher recovery of 1.09% which would be however below the assumed limit of 2% for Grade A2.

Figure 16 on page 70 depicts the overlay of the air and surface recovery rates and shows again the much higher Grade D air recovery rates when compared with the surface recovery rates. The obvious hot spots in Grade D are the documenting rooms (rooms No. 17, 16 and 15) in the south-east of the building, the Grade D men's air lock (GA to Grade D - room No. 6), and Grade D air locks (Grade D to Grade B - men's, women's and unisex air locks - rooms No. 34, 33 and 97 respectively). The obvious hot spots in the APA are the personnel air locks (rooms No. 23, 24, and 96). The Grade D corridor (room No. 9) can also be considered as hot spot because it has the highest air recovery rate in Grade D (82.84%) even though the surface recovery rate does not quite reflect this high air recovery rate. The air and surface recovery rates in the north-west rooms (rooms No. 1 and 66), on the other hand, have lower recovery rates when compared with the other cleanrooms, and could therefore be considered as cold spots.

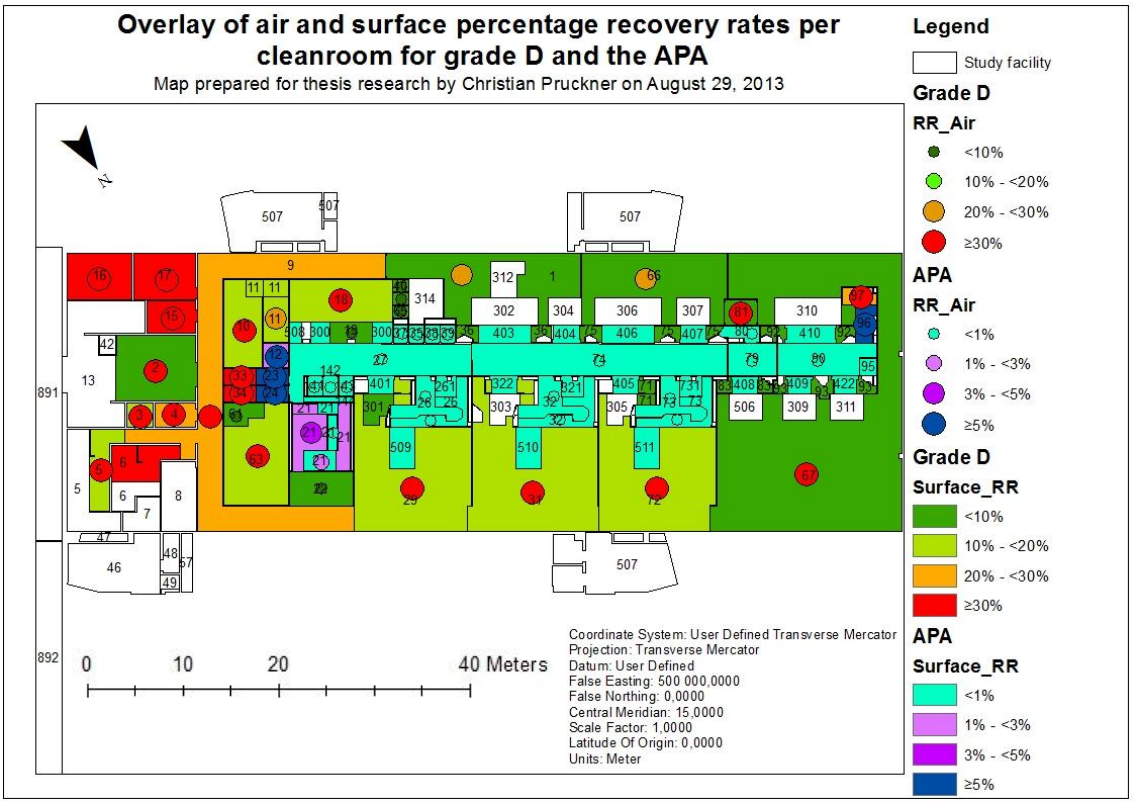

Figure 16: Overlay of air and surface percentage recovery rates per cleanroom for Grade D and the APA

What is unusual is that in the material air lock (GA to Grade D – room No. 2) has quite opposite air and surface recovery rates where the high air recovery rate (35.53%) does not reflect the surface recovery rate (8.85%), even though based on the literature review, it could be expected that a material air lock has high recovery rates from both air and surface monitoring. The hot spot analysis tool (Getis-Ord Gi* tool from ArcToolbox) was deployed in order to establish if the determined hot spot clusters (cleanrooms) have statistically significant (p-value: <0.05) higher combined (air and surface) recovery rates when compared with the other cleanrooms.

Figure 17 on page 71 shows the obtained choropleth map from the hot spot analysis (Getis-Ord Gi*) where the only significant hot spots are the Grade D sterile corridor (room No. 9) with a p-value of <0.001, the men's Grade B air lock (room No. 24) with a p-value of <0.001 as well as the women's Grade B air lock (room No. 23) with a p-value of 0.02. Conversely, other areas, especially the Grade D air locks (rooms No. 6, 33, 34 and 97) and the Grade B unisex air lock (room No. 96) are not statistically significant hot spots according to the Getis-Ord Gi* analysis. Furthermore, the Grade D documenting rooms (rooms No. 17, 16 and 15), the equipment washing room (room No. 10), and the vial washing rooms for sterile filling suites 1-3 (rooms No. 29, 31, and 72) are also not statistically significant hot spots, even though they had significantly higher air recovery rates when compared with the other Grade D cleanrooms.

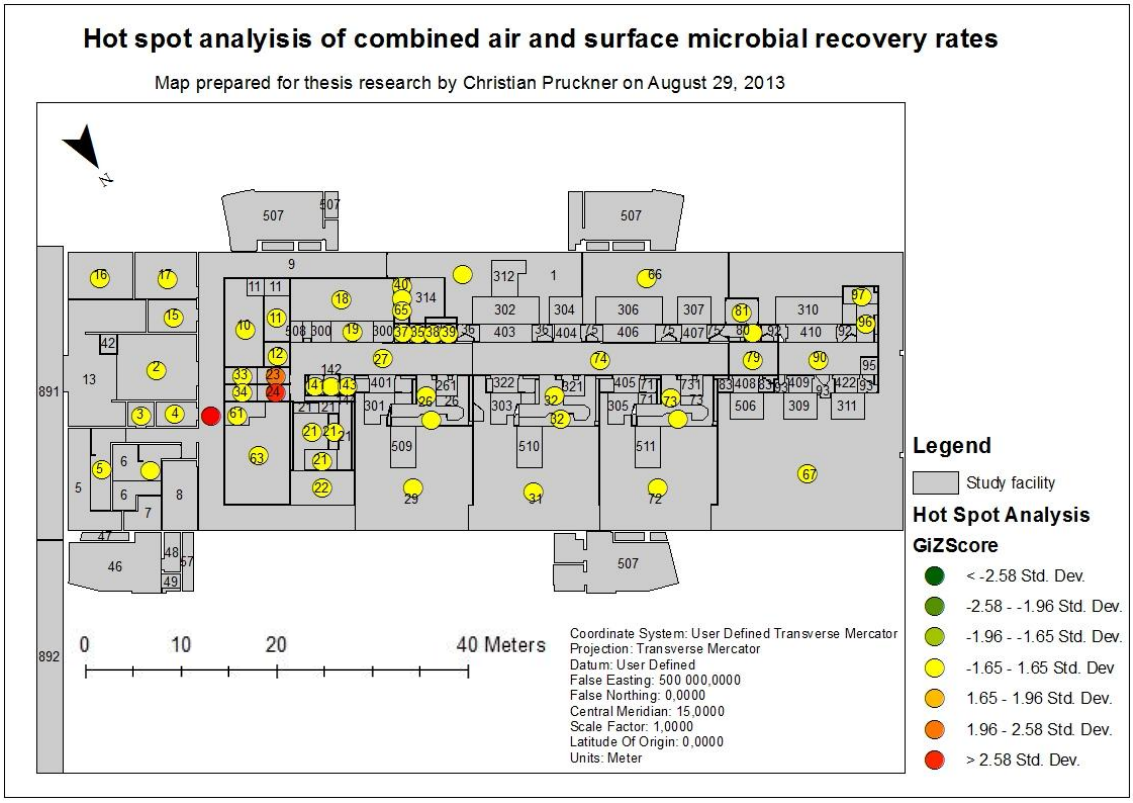

Figure 17: Hot spot analysis of combined air and surface microbial recovery rates

4.2 Have the air and surface microbial counts changed over time based on 3 years of data?

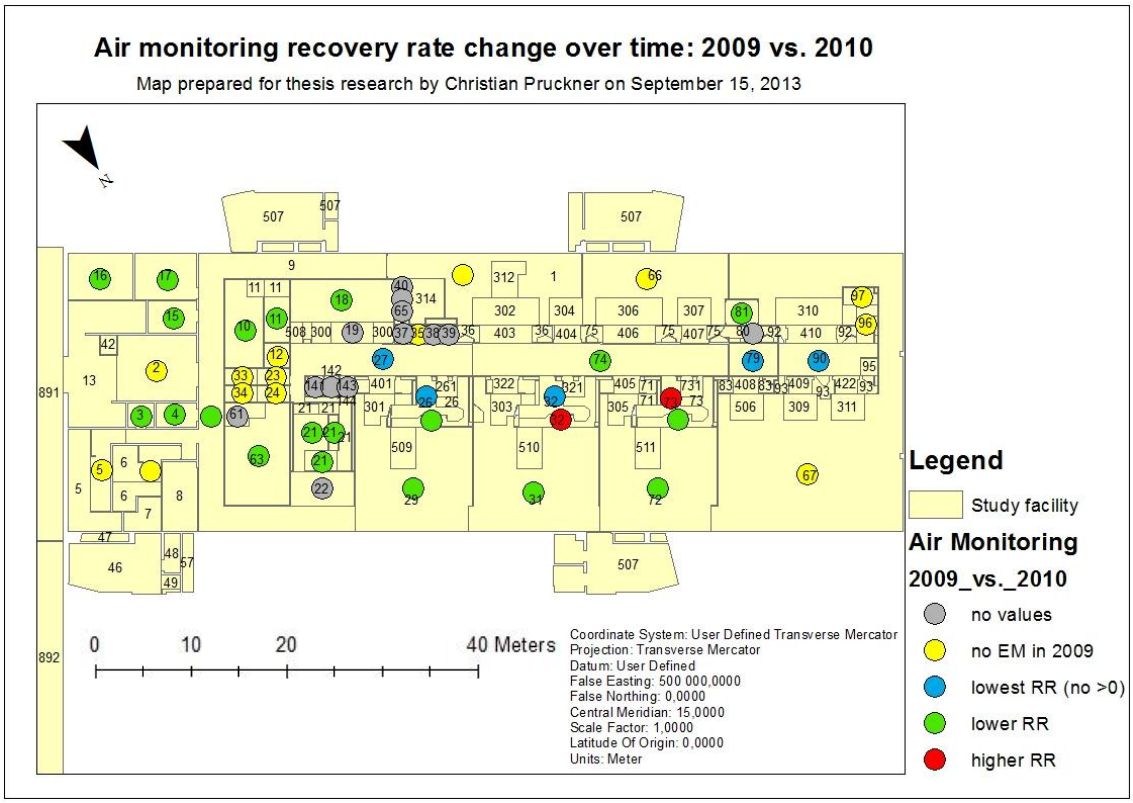

Figure 18: Air monitoring recovery rate change over time: 2009 vs. 2010

Figure 18 on page 72 depicts the air monitoring Recovery Rate (RR) changes between 2009 and 2010. The graphic shows that there were many cleanrooms previously not monitored in 2009. These rooms portrayed in yellow were previously monitored at-rest rather than in-operation and include all Grade D (rooms No. 5, 6, 2, 33, 34 and 97) and Grade B (room No. 12, 23, 24 and 96) air locks, but also the northwest rooms (rooms No. 1, 66 and 67). Some Grade A2 cleanrooms, portrayed in blue, had no >0 values over the three year period of 2009, 2010 and 2011. Overall the majority of cleanrooms had lower RRs in 2010 compared with 2009. Nevertheless, Grade A in cleanroom No. 32 (filling suite 2) and Grade B in cleanroom No. 73 (filling suite No. 3) had slightly higher recovery rates in 2010 when compared with 2009. Some rooms portrayed in grey have currently no air monitoring established, or are monitored at-rest. The RR raw data per annum can be viewed in Appendix 1: Microbiological recovery rate data summary on page 126.

Figure 19 on page 73 depicts the air monitoring RR changes between 2010 and 2011. All previously at-rest monitored rooms have now been monitored in-operation since 2010 when the change took place. The graphic shows that there was an increase in cleanrooms with higher RRs. This is to some extent due to the addition of the previously at-rest monitored cleanrooms that had lower RRs in 2010 when compared with 2011, and partly due to generally higher RRs in other cleanrooms in 2011 when compared with 2010, hence the downwards trend from 2009 to 2010 did not continue. However, a look at the RR raw data in Appendix 1: Microbiological recovery rate data summary on page 126 illustrates that the higher values in the air monitoring RRs between 2010 and 2011 are only minor.

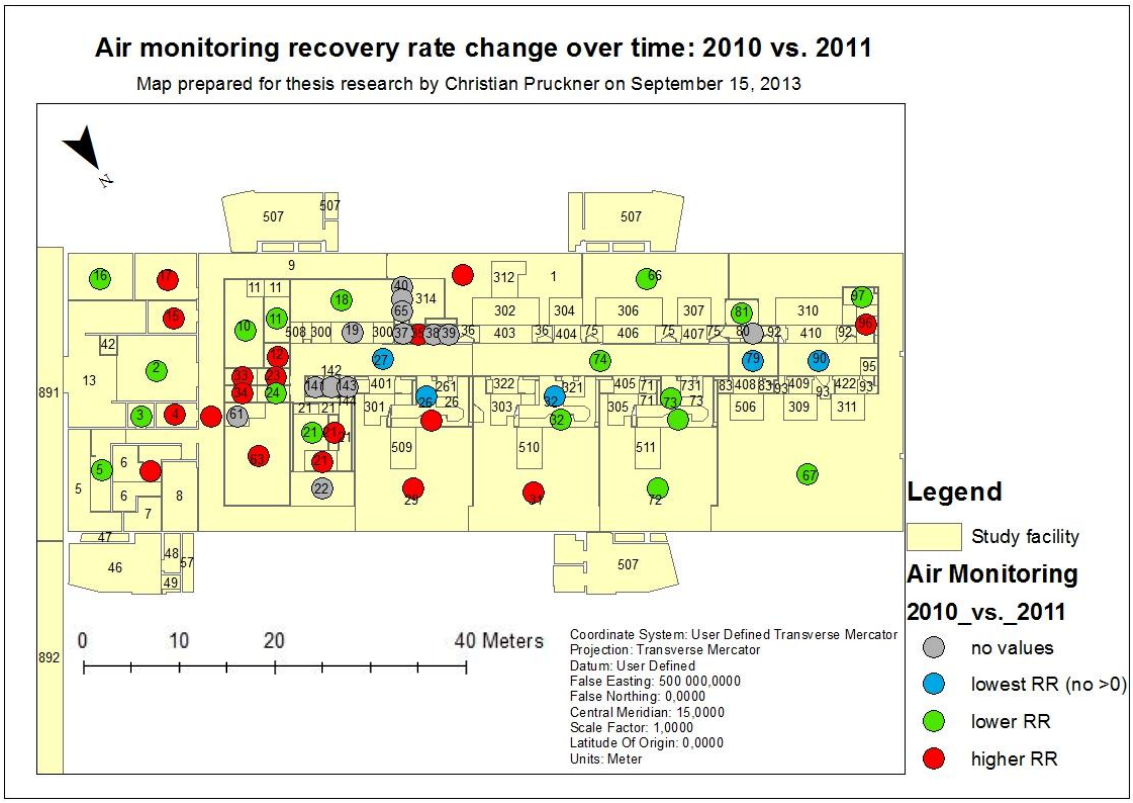

Figure 19: Air monitoring recovery rate change over time: 2010 vs. 2011

Figure 20 on page 74 depicts the air monitoring RR changes between 2009 and 2011, and shows a different picture again, with a lower number of higher RRs in 2011 when compared with 2009. However, the graphic includes many cleanrooms that were not monitored in-operation in 2009. On the other hand, there is only one cleanroom that had a higher RR in 2011 when compared with 2009 (Grade A in room No. 21 – filling suite No. 4) and most RRs of all other cleanrooms have decreased in 2011 when compared with 2009. The Chi Square analysis (χ^2) was deployed to see if these changes in air monitoring RRs are statistically significant.

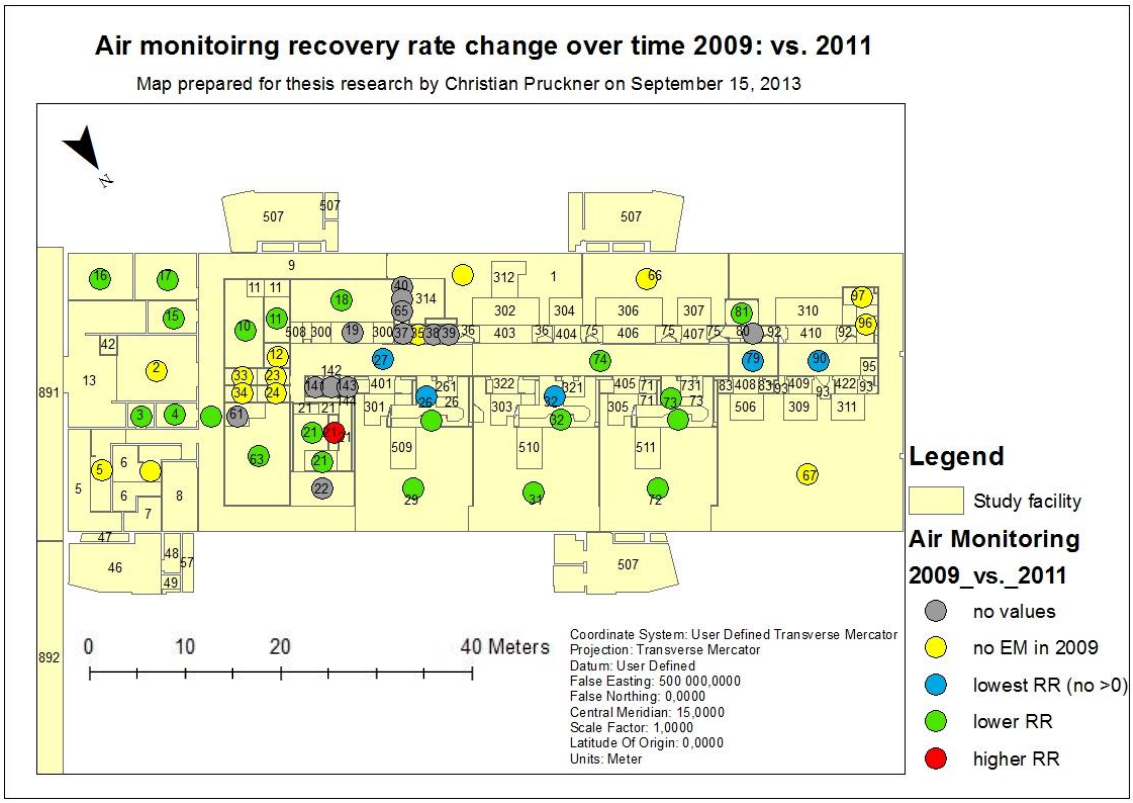

Figure 20: Air monitoring recovery rate change over time: 2009 vs. 2011

Figure 21 on page 75 depicts the results of the χ^2 analysis for the air monitoring RR changes between 2009 (or 2010 if no monitoring in 2009) and 2011. The graphic shows that most air monitoring recovery rate changes are not statistically significant (grey dots). On the other hand, all but two cleanrooms show a reduction in air monitoring RRs. All air monitoring points depicted in green have a statistically significant reduction in RRs between 2009 and 2011 (p-value: <0.05). This includes such cleanrooms as the documenting rooms (room No. 15, 16, 17, 4 and 3), the equipment washing room (room No. 10), the Grade D corridor (room No. 9), the equipment preparation room (room No. 18) and the vial washing rooms for sterile filling suites 1-3 (rooms 29, 31 and 72 respectively) which showed higher air recovery rates in the previous study Question No. 1 Findings Section. Furthermore, the storage room No. 11, the stopper washing/sterilisation room No. 63 and the preparation room No. 81 had also statistically significant reduction in RRs between 2009 and 2011

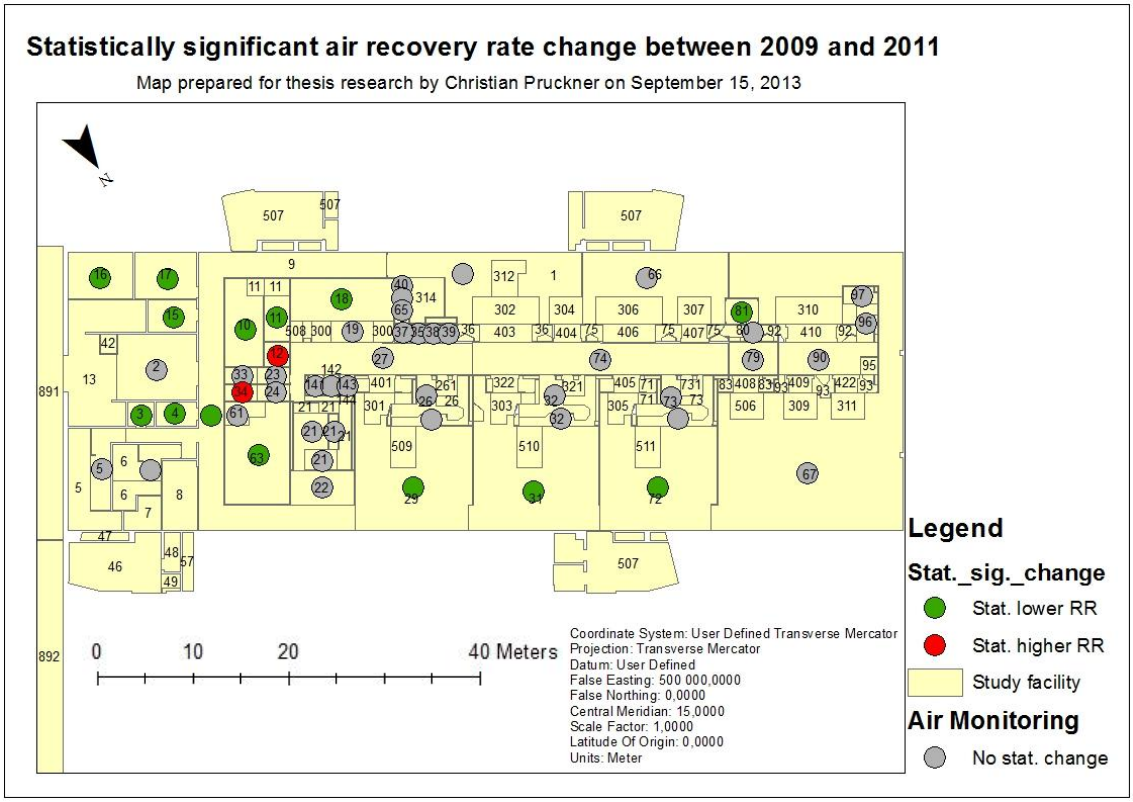

Figure 21: Statistically significant air recovery rate change between 2009 and 2011

Conversely, the air monitoring points in room No. 34 (Grade D men's air lock) and room No. 12 (Grade B material air lock) (both depicted in red) have a statistically significant increase in RRs between 2010 (not monitored in-operation in 2009) and 2011 (p-value: <0.05). The Grade D men's air lock (room No. 34) was also one of the rooms that had higher air and surface RRs (≥40% - combined 2009, 2010 and 2011) in the previous study Question No. 1. Overall, it can be concluded that most of the air

monitoring recovery rates have either remained stable or reduced over the three year period, and only two cleanrooms had an increase in air monitoring recovery rates over this time period.

Figure 22 on page 76 depicts the surface monitoring Recovery Rate (RR) changes between 2009 and 2010. The results go along with the air monitoring RR changes in that there have been many cleanrooms that were not monitored in 2009. These rooms, portrayed in yellow, were previously monitored at-rest rather than in-operation, and include all Grade D (rooms No. 5, 6, 2, 33, 34 and 97) and Grade B (room No. 12 ,23, 24 and 96) air locks, but also the northwest rooms (rooms No. 1, 66 and 67). The depicted 50:50 ratio of cleanrooms/grades that had higher RR to lower RR is, however, different to the air RR change between 2009 and 2010 in that there were only two rooms with a higher air RR. Grade A in cleanroom No. 32 (filling suite 2), which was one of the rooms with a higher air RR in 2010, also has a higher surface RR in 2010. Grade A2 in room No. 73 (filling suite 3), which had a higher air RR in 2010, had a lower surface RR in 2010. As with the air monitoring, some rooms portrayed in grey have currently no surface monitoring established, or are monitored at-rest.

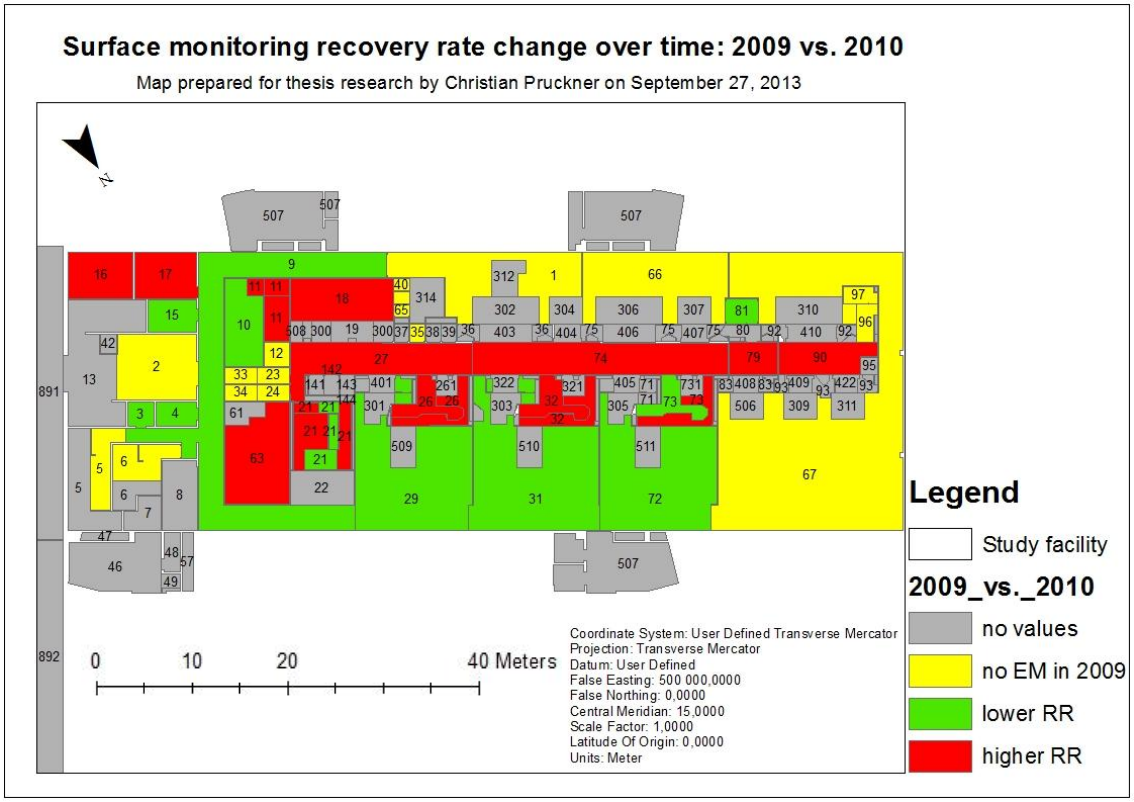

Figure 22: Surface monitoring recovery rate change over time: 2009 vs. 2010

Figure 23 on page 77 depicts the surface monitoring RR changes between 2010 and 2011 and reflects the air monitoring counterpart for the same period. All previously at-rest monitored rooms are now monitored in-operation since 2010 when the change took place. The graphic shows that there has been a slight increase in cleanrooms with higher RRs. This is partly due to the addition of the previously at-rest monitored cleanrooms that had lower RRs in 2010 when compared with 2011 and partly due to generally higher RRs in 2011 when compared with 2010 in other cleanrooms (similar to the air monitoring data). In addition, there are cleanrooms that were incorporated in the in-operation EM programme in 2010 which are only surface monitored (rooms No. 40 and 65 − small equipment storage rooms in the northwest). There are slightly more cleanrooms with higher RRs when compared with rooms with lower RRs. However, a look at the RR raw data in Appendix 1: Microbiological recovery rate data summary on page 126 illustrates that, similar to the results of the air monitoring RRs, the increase in surface monitoring RRs between 2010 and 2011 is only minor.

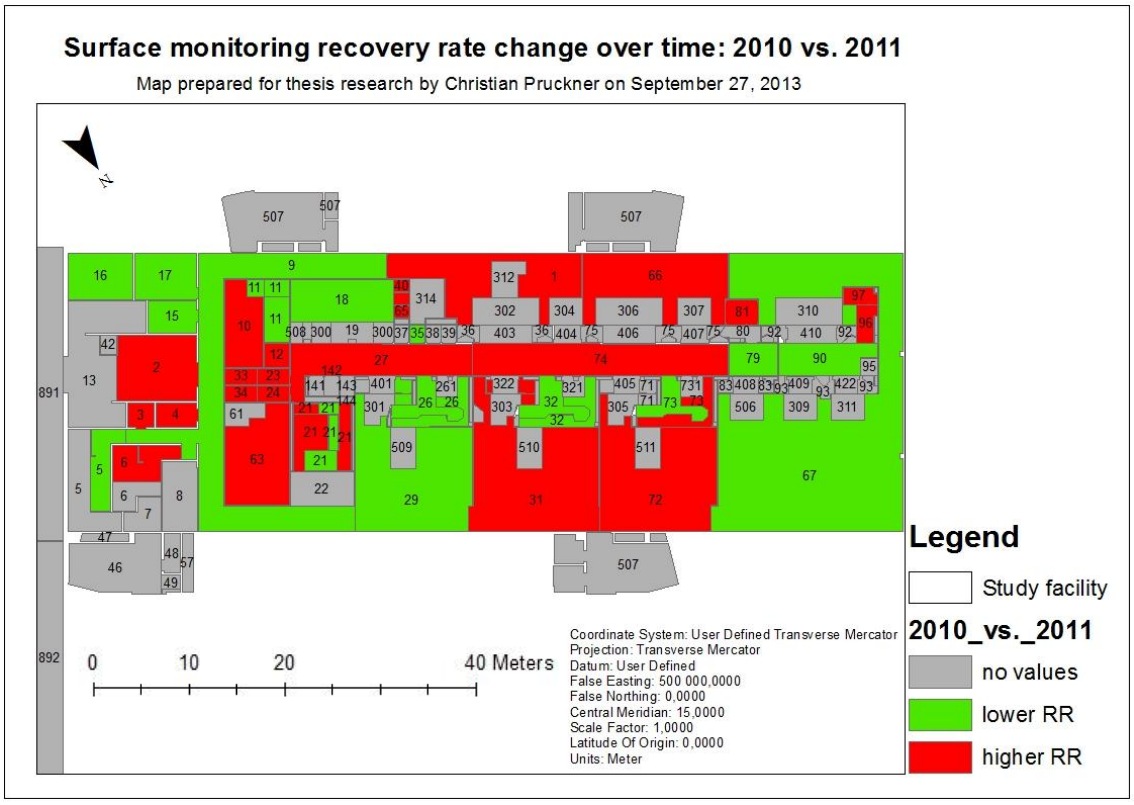

Figure 23: Surface monitoring recovery rate change over time: 2010 vs. 2011

Figure 24 on page 78 depicts the surface monitoring RR changes between 2009 and 2011 which is contrary to its air monitoring RR change counterpart (only one cleanroom grade with higher RR in 2011 – Grade A in room No. 21 = sterile filling suite No. 4) in that there were many more cleanrooms that had higher RRs in 2011 when compared with 2009. Yet this does not include the cleanrooms lacking in-operation monitoring in 2009. Interestingly, the whole APA corridor is among those cleanrooms with higher surface RRs in 2011 when compared with 2009 which was also the case in 2009 vs. 2010 and may show a deteriorating upwards trend in the microbial surface monitoring data. The Chi Square analysis (χ^2) has been deployed to see if these changes in surface monitoring RRs are statistically significant.

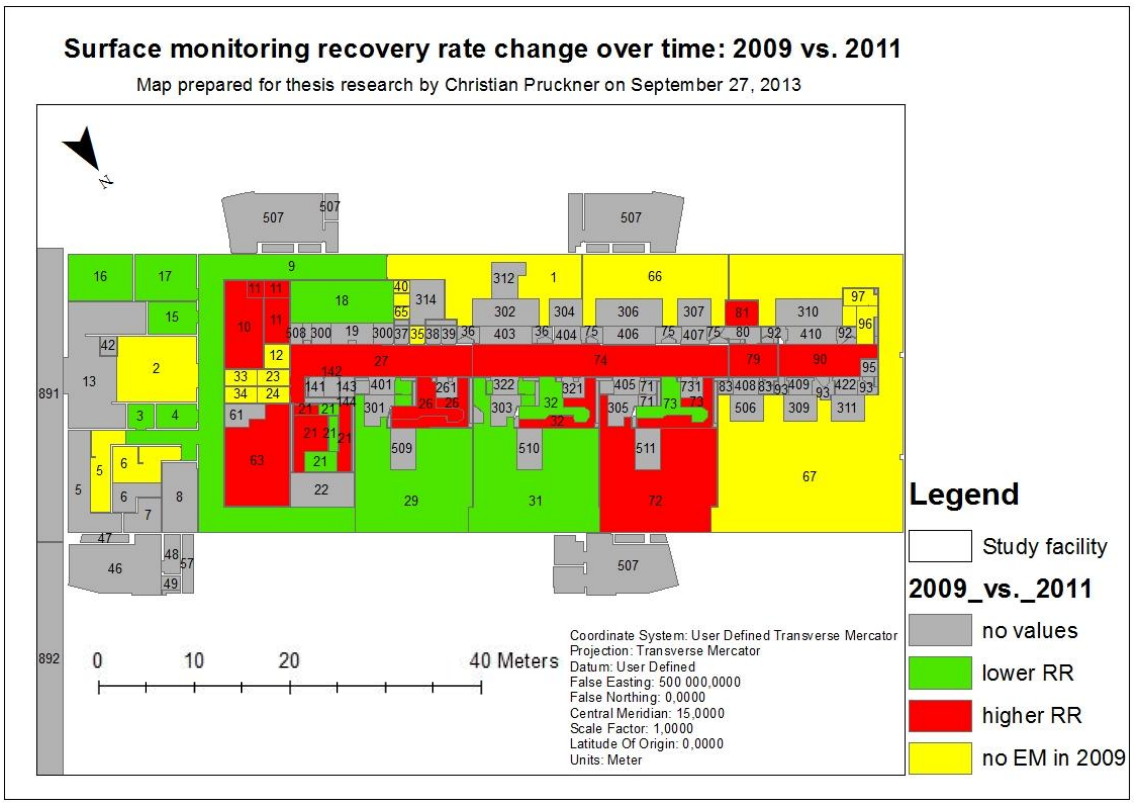

Figure 24: Surface monitoring recovery rate change over time: 2009 vs. 2011

Figure 25 on page 79 depicts the results of the χ^2 analysis for the surface monitoring RR changes between 2009 (or 2010 if no monitoring in 2009) and 2011. The graphic shows that most surface monitoring recovery rate changes are not statistically significant (grey polygons). On the other hand, contrary to the air monitoring results, there were more cleanrooms (or cleanroom grades) with statistically significant higher surface RRs (11 in total - p-value: <0.05) than cleanrooms with statistically significant lower surface RRs (8 in total - p-value: <0.05) in 2011 compared with 2009 (or 2010 where there was no monitoring in 2009).

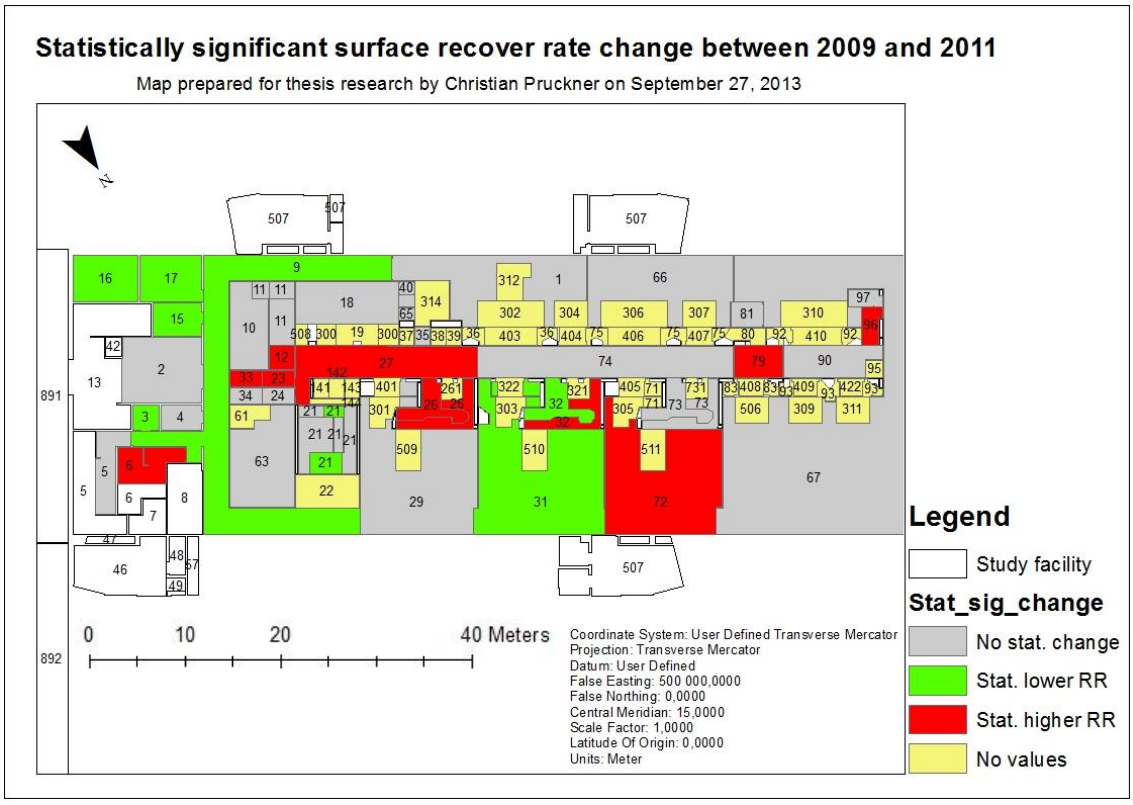

Figure 25: Statistically significant surface recovery rate change between 2009 and 2011

The cleanrooms with statistically significant lower surface RRs include the documenting rooms (room No. 3, 16, 17 and 15, some of which some showed higher surface recovery rates in the previous study, Question No. 1, Findings Section), the Grade D corridor (room No. 9), Grade D preparation area for sterile filling suite 2 (room No. 31), Grade A2 in room 21 (sterile filling suite 4) and Grade A2 in room No. 32 (sterile filling suite 2). The cleanrooms with statistically significant higher surface RRs include the Grade D men's and Grade B unisex air locks (rooms No. 6 and 96 respectively), the Grade D and Grade B women's air locks (rooms No. 33 and 23 respectively) and the Grade B material air lock (room No. 12). The Grade B material air lock is also one of the only two cleanrooms that had a statistically significant higher air RR in 2011 compared with 2010. In addition, part of the APA corridor (rooms No. 27 and 79), Grade A2/A in sterile filling suite No. 1 (room No. 26), the Grade A sterile filling suite No. 2 (room No. 32) and the Grade D vial washing area for sterile filling suite No. 3 (room No. 72) have statistically significant higher surface RRs.

The Grade D and Grade B women's air locks (rooms No. 33 and 23 respectively), the Grade D men's air lock (room No. 6) and the Grade B unisex air lock (room No. 96) had also higher air and surface RRs (≥40% for Grade D and ≥5% for Grade B - combined 2009, 2010 and 2011) in the previous study Question No. 1. Overall it can be concluded that there were more rooms with statistically significant higher surface RRs in 2011 when compared with 2009 than rooms with lower RRs.

Figure 26 on page 80 shows an overly of the statistically significant air and surface recovery rate change between 2009 and 2011 (or 2010 if no monitoring in 2009). The graphic omits for easier understanding the air RRs that had no statistically significant change. Overall the graphic shows that there is a correlation among the increase or decrease in statistically significant (p-value: <0.05) air and surface RRs. There is only one cleanroom (room No. 72) with contrary air and surface RRs.

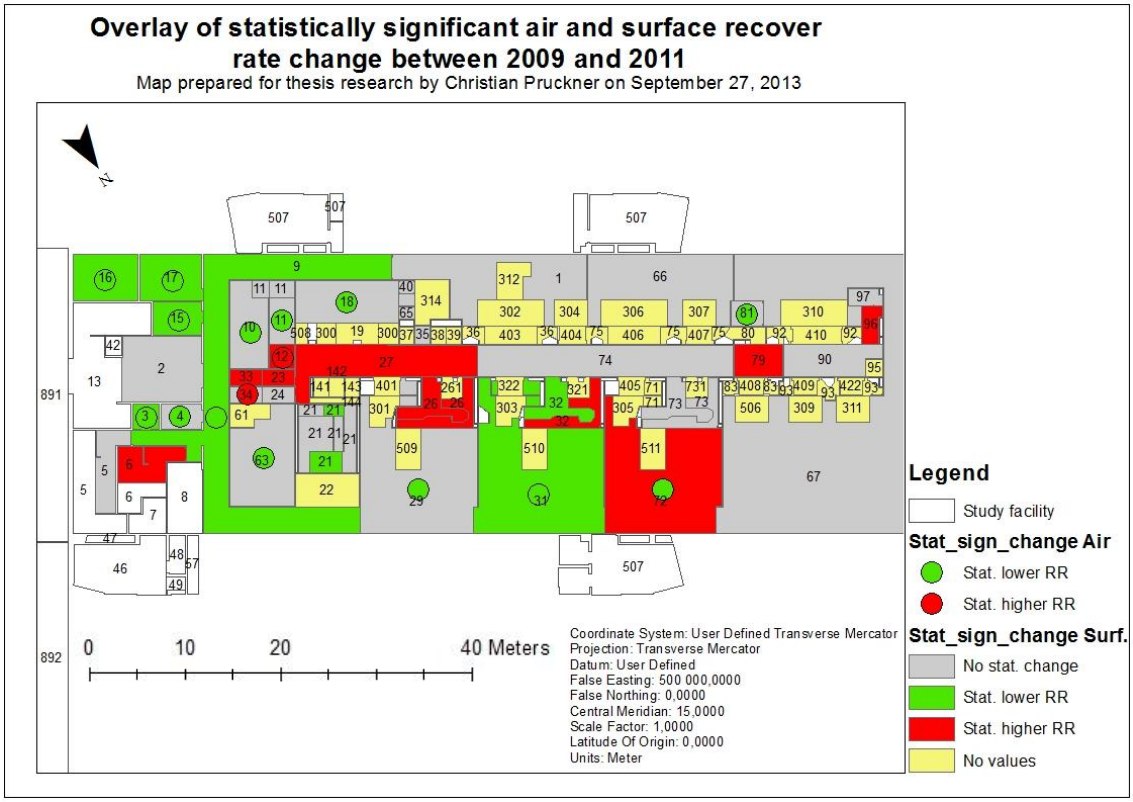

Figure 26: Overlay of statistically significant air and surface recovery rate change between 2009 and 2011

4.3 Is there a certain microbiological flora associated to certain areas of the facility?

The most frequently isolated microbial classes in Grade D (Figure 27 on page 81) of the study facility from 2009-2011 are G+ve cocci C+ (61.73%), most of which are microorganisms found on the human skin (Cundell, 2004), followed by moulds (20.19%) and G+ve rods S- (10.39%), which are found to a lesser extent on the human skin (Cundell, 2004). Microbial classes below 10% in descending order are G+ve rods S+ (3.5%), which are environmental microorganisms found on dust, cellulosic materials, and so on (Cundell, 2004), G-ve rods (3.97%), which are often microorganisms recovered in water (Sandle, 2011), and yeasts. There are no bacteria in the classes G+ve cocci C- and G-ve rods O-, L+, as there are no objectionable organisms in Grade D that would require this class separation.

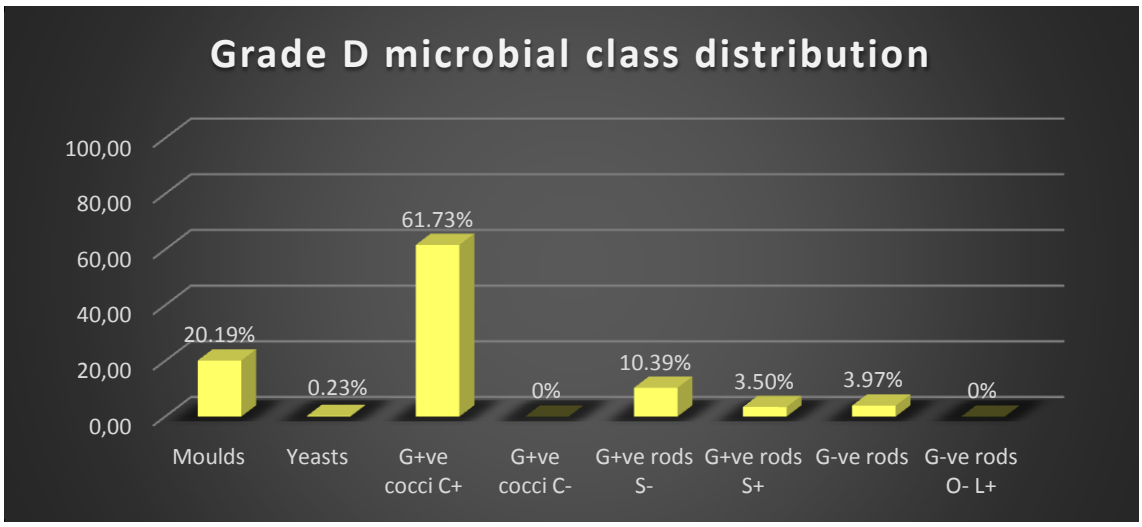

Figure 27: Grade D microbial class distribution

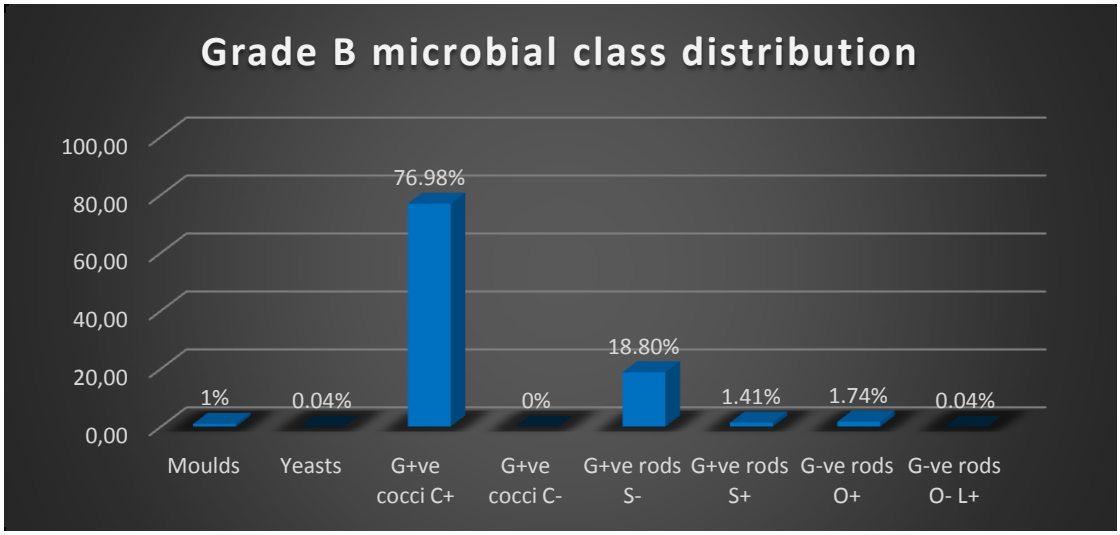

Figure 28: Grade B microbial class distribution

Grade B (Figure 28 on page 81) shows a similar picture, when compared to Grade A, with a higher percentage of G+ve cocci C+ (76.98%) isolates compared with Grade D (61.73%), but significantly less moulds (only 1%). The second highest isolate class in Grade B are G+ve rods S- (18.8%) while microbial classes below 10% in descending order are G-ve rods O+ (1.74%), G+ve rods S+ (1.41%), moulds (1%) and equally G-ve rods O-, L+ (0.04%), which may be bacteria from the coliform group, and yeasts (0.04%). There were no G+ve cocci C- in Grade B during this time period.

The Grade A2 isolate class distribution (Figure 29 on page 82) is similar to Grade B in that there is a high percentage of G+ve cocci C+ (74.94%) isolates and significantly less moulds (only 0.63%). The second highest isolate class are also G+ve rods S- (18.67%). The microbial classes below 10% in descending order are G+ve rods S+ (3.58%) G-ve rods O+ (2.03%), moulds (0.63%) and G+ve cocci C- (0.15%), a group

which some objectionable organisms fall into (e.g. Streptococci). There were no G-ve rods O- L+ or yeasts isolated in Grade A2 during the given time period.

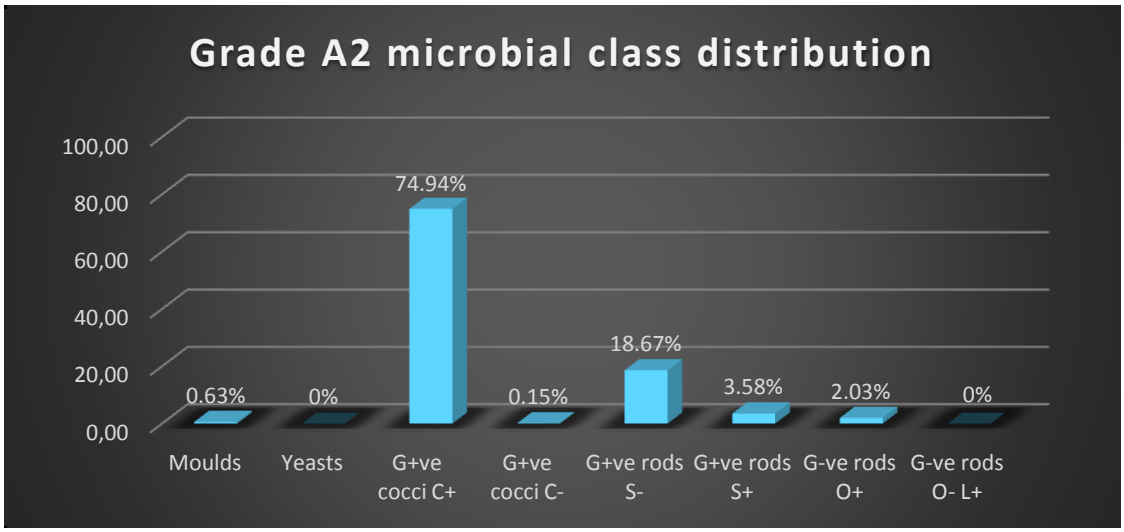

Figure 29: Grade A2 microbial class distribution

The Grade A isolate class distribution (Figure 30 on page 83) is similar to Grade A2, with G+ve cocci C+ being the highest isolated microbial class (64.86%), but the number of G+ve rods S+ isolates is higher (10.51%) compared with Grade A2 (3.58%), and the G-ve rod O+ isolate number is also slightly elevated (5.07%). The number of mould and yeast isolates in Grade A (1.81% and 0.36% respectively) appear also higher compared with the other APA cleanroom grades, but all isolate numbers are in fact lower. A look at the raw data in Appendix 3: Microbial class distribution data on page 141 shows that the higher percentage of microbial isolate classes in Grade A derives from the significantly lower sum of isolates. However, yeasts were recovered in Grade A, while none were recovered in the lower cleanroom class of A2. A combined percentage microbial class distribution from recoveries within the APA for a comparison with the data of the literature review can be seen in Figure 31 on page 83.

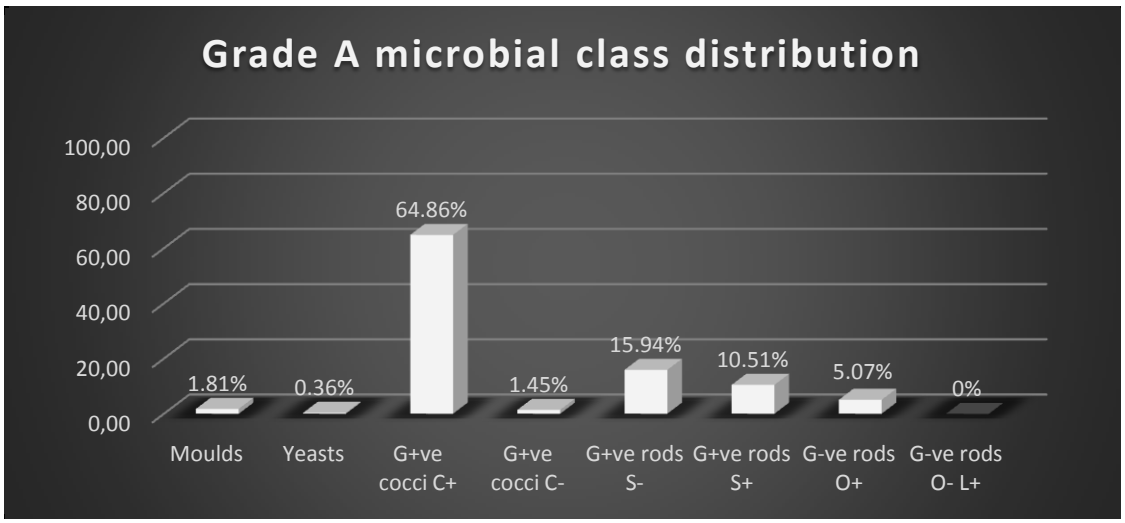

Figure 30: Grade A microbial class distribution

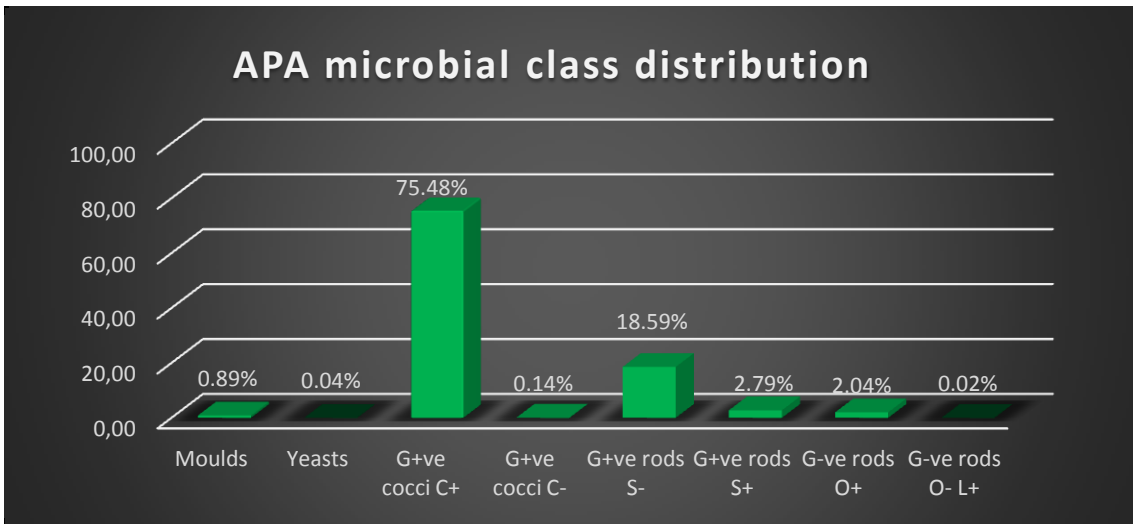

Figure 31: APA microbial class distribution

The recovered top three genera and species separated by microbial classes within all four cleanroom grades do not differ significantly and show an almost identical pattern. The top three G+ve cocci, C+ in ascending order are *Micrococcus luteus*, *Staphylococcus epidermidis* and *Staphylococcus hominis* in all four cleanroom grades. Other species that have a high occurrence in this class and throughout all cleanroom grades include *S. saprophyticus*, *S. capitis*, *S. xylosus*, *M. lylae* and *Kocuria kristinae*, which are all human skin microorganisms (Borriello et al., 2005b). The top three G+ve rods S- in all cleanroom classes include *Brevibacterium spp., Actinomyces spp.* and *Corynebacterium spp.* (*e.g. Corynebacterium jeikeium* or *Corynebacterium macginleyi*), but also *Arthrobacter spp.*, which can be found on human skin and/or mucus membranes as well as the human conjunctiva. Other more common G+ve rods S- isolated in all cleanroom grades include *C. accolens* (a lipophilic *Corynebacterium* which inhabits human ears, eyes, nose and oropharynx), *C. propionquum* (a non-

lipophilic Corynebacterium that inhabits the oropharynx), and *Cellumonas spp*., a soil organism (Borriello et al., 2005b).

The top three G+ve rods S+ in all cleanroom grades are Bacilli, including *Bacillus cereus, Bacillus pumilus*, *Bacillus lentus*, and *Bacillus firmus,* which are all ubiquitous environmental organisms (are found in aerosols, dust and soil), but also *Paenibacillus amylolyticus* a nitrogen-fixing soil microorganism (Borriello et al., 2005a, 2005b). Other G+ve rods S+ microorganisms that were commonly isolated in all cleanroom classes include *B. circulans*, *B. megaterium* and *B. licheniformis*, also ubiquitous environmental microorganisms that can produce toxins in sufficient numbers (Borriello et al., 2005b). Within the top three G-ve rods O+ of the cleanroom grades are *Brevundimonas vesiculari, Sphingomonas paucimobilis*, and *Pseudomonas spp*. including, *Pseudomonas stutzeri* and *Pseudomonas aeruginosa*, but also *Ochrobactrum anthropi* and *Moraxella spp.* Most of these species are found in water or wet environments; for a detailed description of their natural habitat refer to Table 9 on page 85, Table 10 on page 87, Table 11 on page 89, and Table 12 on page 91. Within the top three moulds in the cleanroom grades are *Paecilomyces spp.*, *Aspergillus spp.* and *Penicillium spp.*, which are generally decomposers found in soil and on plant materials (Merz & Hay, 2005).

Within the top three G+ve cocci C- in grades A and B are *Enterococcus faecalis* (which inhabits the human intestinal tract, but can be also found in water, soil, food, plants, and animals), *Streptococcus spp.* (which inhabits the human mucosal membranes and skin), including *Streptococcus parasanguinis* and *Streptococcus* canis (which are all objectionable organisms according to internal procedures listed in Table 3 on page 41) as well as *Aerococcus viridians,* an environmental microorganism (Borriello et al., 2005a). The only yeasts isolated in Grade A during this three year period was *Candida albicans* (which colonises various sites of the human body). *Cryptococcus uniguttulatus* is normally found on plant material, was the only yeast recovered in Grade B. Grade D had only two isolated yeast species, including *Candida famata*, generally found in natural substrates and in several types of cheese (Desnos-Ollivier et al., 2008), and *Rhodotorula mucilaginosa*, *a* common yeast in water-associated environments (Merz & Hay, 2005). The only G-ve rods O-, L+ was *Klebsiella pneumoniae ssp. pneumoniae* recovered in Grade B during a special investigation (with non-routine sampling). This genus belongs to the Enterobacteriaceae family and coliform group and is an objectionable organism, according to internal procedures listed in Table 3 on page 41. The organism can be found in the respiratory tract and bowel of humans, but also in soil and water (Borriello et al., 2005a).

Table 9: Top three identified microorganisms within each microbiological class in Grade A

Microbial class	Rank number	Species or genus	Environmental source and natural habitat
G+ve cocci, catalyse positive (C+)	1	*Micrococcus luteus*	Human skin – present on 20% of human head, legs and arms (Borriello et al., 2005b).
	2	*Staphylococcus epidermidis*	Human skin – most frequently isolated on skin parts with more moisture (Borriello et al., 2005b).
	3	*Staphylococcus hominis*	Human skin (Borriello et al., 2005b).
G+ve rods, spore negative (S-)	1	*Brevibacterium spp.*	Human skin (Borriello et al., 2005a).
	1	*Actinomyces spp.*	Human and animal intestinal surfaces and mucus membranes (mainly in oral cavities) (Borriello et al., 2005a).
	2	*Corynebacterium jeikeium*	Human skin, but also most frequently encountered *Corynebacterium* in clinical specimens (Borriello et al., 2005a).
G+ve rods, spore positive (S+)	1	*Bacillus cereus*	Ubiquitous environmental organism (aerosols, dust, soil), toxin producer, often food pathogen (Borriello et al., 2005a).
	2	*Bacillus pumilus*	Ubiquitous environmental organism (aerosols, dust, soil) (Borriello et al., 2005a).
	3	*Bacillus lentus*	Ubiquitous environmental organism (aerosols, dust, soil) (Borriello et al., 2005b).
G-ve rods, oxidase positive (O+)	1	*Brevundimonas vesicularis*	Freshwater and soil organism (Beilstein & Dreiseikelmann, 2005).
	2	*Sphingomonas paucimobilis*	Opportunistic pathogen, the habitat has not yet been well defined but includes the natural environment (water and soil), but this species has also been isolated in hospital environments and in tap water (Kilic et al., 2007).
	2	*Pseudomonas*	Common on a vast number of materials

		aeruginosa	and in water, forms biofilms and toxins; is an opportunistic pathogen (Borriello et al., 2005b).
Moulds*	1	*Paecilomyces spp.*	Decomposer – soil and plant material, but they have also been isolated from insects. These organisms can cause infection in immunocompromised hosts (Merz & Hay, 2005).
	2	*Aspergillus spp.*	Decomposer – soil and on cellulose materials (Lim, 1998).
G+ve cocci, catalase negative (C-)	1	*Enterococcus faecalis*	Most commonly isolated bacteria from human intestinal tract, but they can be also found in water, soil, food, plants, and animals (Borriello et al., 2005a). Objectionable organism according to internal procedures listed in Table 3 on page 41.
	2	*Streptococcus parasanguinis*	Obligate parasite of the human mucosal membranes. This genus can cause infection when introduced in normally sterile areas of the human body or in immunocompromised patients (Borriello et al., 2005a). Objectionable organism according to internal procedures listed in Table 3 on page 41.
	2	*Aerococcus viridans*	Generally airborne bacterium that can also colonise the human skin (Borriello et al., 2005b).
Yeasts*	1	*Candida albicans*	Colonises various sites of the human body and is a major human pathogen. The species is more rarely isolated from environmental sources (Merz & Hay, 2005).

*Moulds and yeasts are objectionable organisms in Grade A according to internal procedures listed in Table 3 on page 41.

Table 10: Top three identified microorganisms within each microbiological class in Grade A2

Microbial class	Rank number	Species or genus	Environmental source and natural habitat
G+ve cocci, catalase positive (C+)	1	*Micrococcus luteus*	Human skin – present on 20% of human head, legs and arms (Borriello et al., 2005b).
	2	*Staphylococcus epidermidis*	Human skin – most frequently isolated on skin parts with more moisture (Borriello et al., 2005b).
	3	*Staphylococcus hominis*	Human skin (Borriello et al., 2005b).
G+ve rods, spore negative (S-)	1	*Corynebacterium jeikeium*	Human skin, but also most frequently encountered *Corynebacterium* in clinical specimens (Borriello et al., 2005a).
	2	*Actinomyces spp.*	Human and animal intestinal surfaces and mucus membranes (mainly in oral cavities) (Borriello et al., 2005a).
	3	*Brevibacterium spp.*	Human skin (Borriello et al., 2005a).
G+ve rods, spore positive (S+)	1	*Bacillus lentus*	Ubiquitous environmental organism (aerosols, dust, soil) (Borriello et al., 2005b).
	2	*Bacillus pumilus*	Ubiquitous environmental organism (aerosols, dust, soil) (Borriello et al., 2005a).
	3	*Bacillus cereus*	Ubiquitous environmental organism (aerosols, dust, soil), toxin producer, often food pathogen (Borriello et al., 2005a).
G-ve rods, oxidase positive (O+)	1	*Sphingomonas paucimobilis*	Opportunistic pathogen, the habitat has not yet been well defined but includes the natural environment (water and soil), but this species has also been isolated in hospital environments and in tap water (Kilic et al., 2007).
	2	*Brevundimonas*	Freshwater and soil organism (Beilstein

		vesicularis	& Dreiseikelmann, 2005).
	2	*Ochrobactrum anthropi*	A highly versatile bacterium that colonises a wide variety of habitats including polluted soil, plants, insects, nematodes, animals and humans (Romano et al., 2009).
Moulds*	1	*Aspergillus spp.*	Decomposer – soil and on cellulose materials (Lim, 1998).
	2	*Aspergillus vesicolor*	Decomposer – found in soil and on cellulose materials (Lim, 1998), can cause aspergillosis in human nails (Merz & Hay, 2005).
	2	*Paecilomyces spp.*	Decomposer – soil and plant material, but they have also been isolated from insects. These organisms can cause infection in immunocompromised hosts (Merz & Hay, 2005).
G+ve cocci, catalase negative (C-)	1	*Streptococcus parasanguinis*	Obligate parasite of the human mucosal membranes and upper respiratory tract (Borriello et al., 2005a). Objectionable organism according to internal procedures listed in Table 3 on page 41.
	1	*Streptococcus canis*	Obligate parasite of the human and animal mucosal membranes and upper respiratory tract (Borriello et al., 2005a). Objectionable organism according to internal procedures listed in Table 3 on page 41.
	1	*Aerococcus viridans*	Generally airborne bacterium that can also colonise the human skin (Borriello et al., 2005b).

* Moulds are objectionable organisms in Grade A2 according to internal procedures listed in Table 3 on page 41.

Table 11: Top three identified microorganisms within each microbiological class in Grade B

Microbial class	Rank number	Species or genus	Environmental source and natural habitat
G+ve cocci, catalase positive (C+)	1	*Micrococcus luteus*	Human skin – present on 20% of human head, legs and arms (Borriello et al., 2005b).
	2	*Staphylococcus hominis*	Human skin (Borriello et al., 2005b).
	3	*Staphylococcus epidermidis*	Human skin – most frequently isolated on skin parts with more moisture (Borriello et al., 2005b).
G+ve rods, spore negative (S-)	1	*Corynebacterium jeikeium*	Human skin, but also most frequently encountered *Corynebacterium* in clinical specimens (Borriello et al., 2005a).
	2	*Actinomyces spp.*	Human and animal intestinal surfaces and mucus membranes (mainly in oral cavities) (Borriello et al., 2005a).
	3	*Corynebacterium macginleyi*	Human conjunctiva and skin (Borriello et al., 2005b), can cause eye infections (Borriello et al., 2005a).
G+ve rods, spore positive (S+)	1	*Bacillus cereus*	Ubiquitous environmental organism (aerosols, dust, soil), toxin producer, often food pathogen (Borriello et al., 2005a).
	1	*Bacillus lentus*	Ubiquitous environmental organism (aerosols, dust, soil) (Borriello et al., 2005b).
	1	*Bacillus firmus*	Ubiquitous environmental organism (aerosols, dust, soil) (Borriello et al., 2005b).
G-ve rods, oxidase positive (O+)	1	*Sphingomonas paucimobilis*	Opportunistic pathogen, the habitat has not yet been well defined but includes the natural environment (water and soil), but this species has also been isolated in hospital environments and in tap water (Kilic et al., 2007).
	2	*Pseudomonas*	Ubiquitous water microorganism that

		stutzeri	can be found in surface waters, aquifers, seawater, vegetation and soil, but also on tertiary water treatment devices (Borriello et al., 2005b) (reverse osmosis – used to treat water for pharmaceutical production, filter beds, membranes).
	3	*Moraxella spp.*	Moraxella species are parasite of the mucus membrane of humans and other warm blooded animals (Krieg & Holt, 1994) and are normally present in the mucous membranes, oropharynx, genital tract and on skin (Public Health Agency of Canada, 2011).
Moulds*	1	*Aspergillus spp.*	Decomposer – soil and on cellulose materials (Lim, 1998).
	2	*Penicillium spp.*	Decomposer – soil and on cellulose materials (Lim, 1998).
	3	*Paecilomyces spp*	Decomposer – soil and plant material, but they have also been isolated from insects. These organisms can cause infection in immunocompromised hosts (Merz & Hay, 2005).
Yeasts*	1	*Cryptococcus uniguttulatus*	*Cryptococcus spp.* are mainly found in substrates of plant and animal origin and in dust (Leite et al., 2012).
G-ve rods, oxidase negative and lactose positive (O-, L+)	1	*Klebsiella pneumoniae ssp. pneumoniae*	This genus belongs to the Enterobacteriaceae family and coliform group. *Klebsiella pneumoniae ssp. pneumoniae* can be found in the respiratory tract and bowl of humans, but also in soil and water. The organism can cause bronchopneumonia and sepsis in patients (Borriello et al., 2005a). Objectionable organism according to internal procedures listed in Table 3 on page 41.

* Moulds and yeasts are objectionable organisms in Grade B according to internal procedures listed in Table 3 on page 41.

Table 12: Top three identified microorganisms within each microbiological class in Grade D

Microbial class	**Rank number**	**Species or genus**	**Environmental source and natural habitat**
G+ve cocci, catalase positive (C+)		*Micrococcus luteus*	Human skin – present on 20% of human head, legs and arms (Borriello et al., 2005b).
		Staphylococcus epidermidis	Human skin – most frequently isolated on skin parts with more moisture (Borriello et al., 2005b).
		Staphylococcus hominis	Human skin (Borriello et al., 2005b).
G+ve rods, spore negative (S-)	1	*Brevibacterium spp.*	Human skin (Borriello et al., 2005a).
	2	*Actinomyces spp.*	Human and animal intestinal surfaces and mucus membranes (mainly in oral cavities) (Borriello et al., 2005a).
	2	*Arthrobacter spp.*	One of the most common isolated soil microorganisms, but some species can also colonise the human skin (Borriello et al., 2005b).
G+ve rods, spore positive (S+)	1	*Paenibacillus amylolyticus*	Nitrogen-fixing soil microorganism (Borriello et al., 2005a).
	2	*Bacillus cereus*	Ubiquitous environmental organism (aerosols, dust, soil), toxin producer, often food pathogen (Borriello et al., 2005a).
	2	*Bacillus lentus*	Ubiquitous environmental organism (aerosols, dust, soil) (Borriello et al., 2005b).
G-ve rods, oxidase negative (O+)	1	*Sphingomonas paucimobilis*	Opportunistic pathogen, the habitat has not yet been well defined but includes the natural environment (water and soil), but this species has also been isolated in hospital environments and in tap water (Kilic et al., 2007).

	2	*Moraxella spp.*	Moraxella species are parasite of the mucus membrane of humans and other warm blooded animals (Krieg & Holt, 1994) and are normally present in the mucous membranes, oropharynx, genital tract and on skin (Public Health Agency of Canada, 2011).
	3	*Brevundimonas vesicularis*	Freshwater and soil organism (Beilstein & Dreiseikelmann, 2005).
Moulds	1	*Aspergillus spp.*	Decomposer – soil and on cellulose materials (Lim, 1998).
	2	*Paecilomyces spp.*	Decomposer – soil and plant material, but they have also been isolated from insects. These organisms can cause infection in immunocompromised hosts (Merz & Hay, 2005).
	2	*Penicillium*	Decomposer – soil and on cellulose materials (Lim, 1998).
Yeasts		*Candida famata*	Generally found in natural substrates and in several types of cheese. This organism is a rare human fungal pathogen (Desnos-Ollivier et al., 2008).
		Rhodotorula mucilaginosa	A common yeast in water associated environments (Merz & Hay, 2005).

4.4 What is/are the potential contamination route(s) in the cleanrooms?

Material and personnel flow are the most significant microbial contamination routes in Grade D and the APA, as the validated physical parameters (differential pressure, HEPA filtered air supply, etc.) and the validated cleanroom status make other microbial entry pathways into the cleanrooms almost impossible. In addition, the microbial entry routes in the APA through the autoclave, sterilisation tunnels and peracetic acid lock are validated (via loading schemes, sterilisation − 6 log reduction, biodecontamination − 3 log reduction), hence, material and personnel air locks are the only significant microbial contamination routes. This is also supported by Figure 16 on page 70 and Figure 17 on page 71, which show that the personnel air locks and to some extent the material air locks have the highest RRs, plus two hot spots (Grade B men's and women's air locks). This conclusion that the material and personnel flow are the only significant possible microbial contamination routes is also supported in ISO 14644-5 (2004) and by section <1116> of the USP, which states that the contamination risk in the cleanroom environment correlates with the movement of personnel within the cleanrooms (The United States Pharmacopeial Convention, 2012/a).

Lampe (2013) supports this theory by stating that personnel is the most important factor and greatest source of microbial contamination within the cleanroom, especially during manual aseptic processing in any pharmaceutical manufacturing operation. It is no surprise that the Grade D corridor (room No. 9), which has to be passed through by personnel coming from the material and personnel air locks, had the highest air RR and was the most significant hot spot (see Figure 17 on page 71). The material and personnel flow is illustrated in Figure 32 on page 94 and shows the correlation between the movement of personnel and high RRs, including hot spots. For example, the documenting areas where only personnel are located have high RRs, whereas the areas on the northwest side of the building, which are less frequented by personnel, generally have lower RRs. In addition, the Grade D and Grade B personnel air locks, including the unisex air locks which are isolated in the north-west corner of the building, have high RRs. Furthermore, the most commonly isolated microbial class in all cleanroom grades is G+ve cocci C+ which are microorganisms found on the human skin (Cundell, 2004). This supports the theory that personnel are the highest contributing factor for microbial contamination of the cleanrooms. Items brought into the APA through the Grade B material air lock (room No. 12) are disinfected with approved disinfectants (validated for microbial reduction). However, it often depends on the personnel as to whether the disinfection is executed properly (depending on exposure time, coating, etc.) and the surface and construction of the material that is brought in.

Therefore, the Grade B material air lock may be a contributing factor to contaminants being brought into the APA.

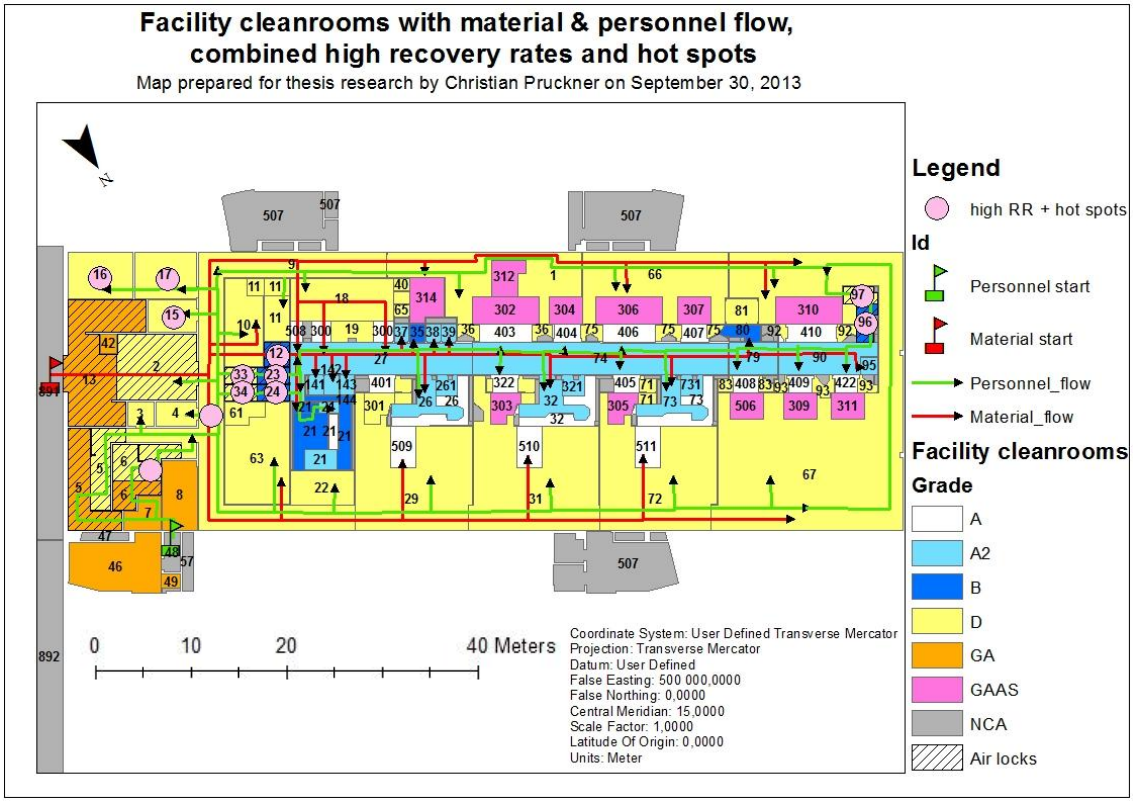

Figure 32: Facility cleanrooms with material & personnel flow, combined high recovery rates and hot spots

Table 13 on page 95 was generated to narrow down and list additional cleanroom contamination risks resulting from material and personnel flow, as well as other contributing factors derived from the literature review. The table lists potential microbial contamination root causes in cleanrooms, including pro and contra arguments to derive a risk score from 1-3 (1 being the highest risk) for the implementation of actions based on risk and urgency (using, for example, balanced scorecards for time and resource planning).

Table 13: List of cleanroom contamination risks due to material and personnel flow as well as other contributing factors derived from the literature review

Cause	Pro	Contra	Score
Equipment is not kept in the APA in order to minimise the risk of introducing contaminants. (ISO 13408-1, 2008).	Some machine parts are taken out of the APA and are brought back in through the material air lock, as they cannot be sterilised or biodecontaminated via peracetic acid, or H2O2, e.g. MAS-device and conveyor screw.	These parts remain most of the time within the APA and are brought out only occasionally.	2
Inadequate initial risk assessment in terms of Identification of contamination risks, including origins, routes, microbial proliferation, contamination, and adequate removal of microbes (ISO 13408-1, 2008).	The origins and microbial proliferation of microorganisms are not fully covered by risk assessment.	The facility has a risk assessment and Failure Mode and Effects Analyses (FMEAs) which lack only certain specificity.	2
Lack of maximum personnel occupancy in Grade D (and C) cleanrooms (ISO 14644-5, 2004).	There is no maximum personnel occupancy rate in Grade D (and C) cleanrooms. The currently established rationale that describes why no maximum personnel occupancy rate in grades C and D is not necessary was not accepted during an internal audit. Therefore, the currently established rationale is not valid and this is why a maximum personnel occupancy	The number of personnel is fairly constant and kept to a minimum most of the time. However, there are more people in the cleanrooms during shift changes, maintenance and internal/external inspections.	2

	number should be established in grade C/D cleanrooms		
Inadequate exit (undressing) procedures for personnel (ISO 14644-5, 2004)	There is no description in the SOP regarding where to place the worn cleanroom gowning items. This may lead to air lock microbial contamination by placing those items on the floor or bench. The Grade D air locks leading to the APA have high air and surface recovery rates.	Only the socks and shoes may be contaminated by stepping on those areas that have been contaminated, but it is a potential carry-over source of microorganisms.	1
Inadequate disposal of waste in personnel air locks (ISO 14644-5, 2004).	The packaging material waste that has been touched with bare and potentially microbial contaminated gloved hands is left in the "waste shelf" for the next person to take out; this leads to a contamination of the shelf.	Proper aseptic technique during dressing with disinfection procedures should reduce the risk of microbial contamination of the personnel.	2
Lack of training by trainers who have some sort of teaching qualification (e.g. train the trainer) (Schniepp, 2013). Lack of periodic retraining (Lampe, 2013) and reassessment of training effectiveness (Pharmaceutical Inspection Convention, 2009/a).	The on-the-job training of production personnel is performed by more experienced staff members, who in most cases do not have any sort of trainer qualification. There is no periodic retraining of aseptic techniques. The aseptic behaviour in Grade D is only checked during infrequent reality	The aseptic behaviour during presence in grades A2/B and staged interventions in Grade A of filling lines is assessed during media fills by quality personnel at least twice a year. Personnel have to be re-qualified for the APA by participating at a media fill once a year where they are contact	3

	checks, internal and external GMP audits by the quality department.	plate monitored after Grade A interventions and before leaving the APA. An experienced staff member who trains a new employee may do a good job of training without a formal qualification.	
Lack of health examinations prior to employment or access to cleanroom for some personnel who have access to any area where the drug product may be exposed in the course of its manufacture. (Health Canada, 2009).	The personnel of the quality department, maintenance personnel and any external personnel (e.g. contractors) who have access to cleanrooms where the product may be exposed in the course of its manufacture are not health-examined prior to employment or prior to approval to enter a cleanroom area.	The stated personnel is less commonly present in cleanrooms than production and GMP-support personnel and they do not handle open product.	3
Doors are left open during air lock cleaning between Grade B and the APA corridor (Grade A2) and this may transfer non-viable and viable particles between rooms (ISO 14644-5, 2004).	Differential pressure alarms have occurred due to this practice with negative pressure in the adjacent cleanroom.	The cleaning of the air locks is mainly performed after production.	2
Not enough Heating Ventilation and Air Conditioning (HVAC) capacity to handle seasonal fluctuations in humidity and temperature	The temperature and humidity during hot summer periods can exceed the action levels of those physical parameters.	Every significant deviation from the required physical parameters is risk assessed in a non-conformance report.	3

(Sartain & Polarine, 2011).	Microorganisms - especially moulds - could proliferate in these conditions.	This issue applies only to a short period during summer time.	
Sporicidal disinfectants corrode stainless steel surfaces (Sartain & Polarine, 2011).	Rust reduces the effectiveness of disinfectants.	There is only a small number of stainless steel surfaces with the cleanrooms that have started to rust.	3
Particle shedding materials (e.g. cardboard) are not vacuumed with an appropriate cleanroom vacuum cleaner with built in HEPA or ULPA filters (ISO 14644-5, 2004) upon entering the GA side of the material air lock (before unpacking).	Cardboard and other particle shedding material can disperse viable particles (especially mould spores) as well as non-viable particles during unpacking, these could settle on the Grade D side of the one-chamber material air lock.	The surface monitoring results of the Grade D material air lock do not show high microbial recovery rates, see Figure 14 on page 67.	2
There is no neutral or negative differential pressure in the area of smaller maintenance work during production times and no additional EM (European Commission, 2008; ISO 14644-5, 2004).	Non-viable and viable articles from the area of maintenance work can disperse to adjacent cleanroom areas.	A sealed partition is put up during most maintenance works.	2
Items routinely brought in and removed from the cleanroom by personnel, such as batch records, pens, hand tools and other kinds of small portable utensils can be a source of contamination to the cleanroom (ISO 14644-5, 2004).	These items are brought into cleanrooms on a regular basis.	These items are only brought into Grade D (and C) areas, while items destined for the APA have to be sterilised.	2
Inadequate material and personnel air lock cleaning/disinfection frequencies during times of	Cleaning/disinfection frequencies in material and personnel air locks are often not	Air locks are cleaned/disinfected at least once a day.	1

high throughput (ISO 14644-5, 2004).	adjusted according to throughput and this may contribute to a carry-over of microorganism in adjacent cleanrooms of higher grades.		
Interventions by personnel in Grade A during filling can contaminate the sterile product with microorganisms (The United States Pharmacopeial Convention, 2012/a).	Interventions in Grade A pose one of the greatest risks to product contamination.	Routine interventions and new interventions during normal fillings in Grade A have to be simulated during media fills and assessed by quality staff. SOPs describe the correct aseptic interventions.	2

5 Discussion

5.1 Which areas in the manufacturing cleanroom facility have higher air and surface microbial counts?

The higher recovery rates in the air monitoring data compared with surface Recovery Rates (RRs) could be explained by the lower number of air sampling points than surface sampling points. There is only one air sampling point for rooms with ≤50m^2 in size in this facility, while there may be 30 and more surface sampling points within this room size. The Grade D air is expected to have a lot more microorganisms when compared with the APA, due to both the turbulent air flow and the fact that the air monitoring action level in Grade D allows ≤200 cfu/m^3. Therefore the number of sampling points makes a big difference, as it can be concluded that the probability of air samples with any microbial recovery is much higher if there are only one or two sampling points, compared with 30 sampling points or more. The number of sampling points is therefore a bias especially in the air monitoring data in terms of the air monitoring recovery rates appearing much higher than they would probably be if more air sampling points were monitored. However, the number of sampling points for air and surface monitoring have been set during the initial cleanroom validation and could only be changed during a cleanroom revalidation by altering the risk assessment in the validation master plan that provides guidance on the number of air sampling points for the cleanroom validation. Furthermore, the phenomenon that the air recovery rates are significantly higher compared with the surface recovery rates in some cleanrooms, such as the material air locks (rooms No. 2 and 12), could be attributed to a higher cleaning/disinfection frequency when compared with the other cleanrooms. Nevertheless, USP section <1116> states the same recovery rate limits for air and surface (and personnel), hence the recovery rates should be similar. Since there is no recovery rate limit set for Grade D, it is not a regulatory requirement. In this case it may be enough to utilise the current trending regime using control charts for Grade D rather than the recovery rate tool. In addition, the more stringent recovery rate threshold of <2 (which is rounded down half way between the <1% for Grade A and <5% for Grade B) or even <3 for ISO 6 could be established for Grade A2 since Grade A2 is not an official grade, but lies between grades A and B.

The high surface and air percentage recovery rates in the Grade D documenting rooms (rooms No. 17, 16 and 15) can be expected, as these rooms are occupied and frequented by personnel all day for documentation and administration duties. These cleanrooms could be a contamination source for other cleanrooms, as people could drag viable particles to other areas in Grade D and the APA. This reflects the USP section <1116> statement that the contamination risk within the cleanrooms correlates

with the movement of personnel (The United States Pharmacopeial Convention, 2012/a). It also correlates with ISO 14644-5 (2004) which states that many items used by personnel that are routinely brought in and removed from the cleanroom by personnel (such as batch records, pens, hand tools and other kinds of small portable utensils) can be a source of contamination to the cleanroom. These items, especially documents that are contaminated with microorganisms and that cannot be easily sanitised, are brought in from the GA or non-classified area to the documenting rooms, and subsequently contaminate these rooms, along with others, due to microorganism dispersion by personnel. Therefore inadequate material flow from the GA to the Grade D cleanrooms should be avoided.

The higher air recovery rates in the northeast side of the building, where the Grade D vial washing areas for sterile filling suites 1-3 are located, could be explained by the higher personnel numbers present compared with the northwest of side of the building, and reflects the USP section <1116> statement that contamination risk within the cleanrooms correlates with the movement of personnel and that the variation of viable particle recovery rates depends on the activities being conducted (The United States Pharmacopeial Convention, 2012/a). Personnel air locks are generally more exposed to microorganisms as a result of the gowning procedure which is also stated in ISO 14644-5 (2004). The fact that that the Grade D men's air locks and also Grade B men's air locks have higher recovery rates compared with the women's air locks is an anomaly that may be explained by the higher number of male maintenance technicians that are sporadically in Grade D and the APA for maintenance work. These staff members are less experienced in cleanroom gowning, as they do not enter the cleanrooms on a daily basis and they often need to take tools with them which may be difficult to disinfect. In this regard, ISO 14644-5 (2004) states that maintenance work is a major contamination factor in the cleanrooms for viable and non-viable particles.

The Grade B personnel air locks have much higher air and surface microbial counts compared with the other cleanrooms within the APA. This could be explained by the increased exposure of the skin and hence shedding of microorganisms, as people have to strip down to their underwear before getting dressed in the cleanroom gowning. The air and surface recovery rates in the Grade B air locks (with the exception of the surface RR in the material air lock) are also above the USP section <1116> threshold (<5%) and therefore breach a cGMP regulatory requirement. However, the data set does not show an imminent issue with higher recovery rates in the adjacent APA cleanrooms. Even so, microorganisms may be dragged from the Grade B air locks to other cleanrooms within the APA and even in the critical processing zone (Grade A)

due to higher RRs in the Grade B air locks. Reducing the hazard of viable particle dispersion by personnel in the Grade B personnel air locks may require the use of cleanroom undergarments (underneath the cleanroom garment), as recommended by Sandle (2011), among other measures.

The results of the hot spot analysis identified the Grade D corridor (room No. 9) and the two Grade B personnel air locks (men's and women's air locks - room No. 24 and 23 respectively) as the only statistically significant hot spots. The Grade D corridor (room No. 9) seems to be centre of the highest microbiological recovery rate exposure in Grade D, and adding to this, it is also the cleanroom with the highest air recovery rate. The reason why the documenting cleanrooms and other areas in Grade D with higher combined recovery rates were not identified as statistically significant hot spots is most likely that these areas were less influenced by surrounding cleanrooms that had also higher combined recovery rates and because the combined recovery rates in these cleanrooms were lower than the combined recovery rate in the Grade D corridor (room No. 9) hot spot. The Grade B men's (room No. 24) and women's air locks (room No. 23) form the hot spot cluster in the APA. The unisex air lock (room No. 96) may not be a statistically significant hot spot due to its isolated location among APA cleanrooms with low recovery rates. The material air lock, on the other hand, seems to have a combined recovery rate that is too low to be part of the APA hot spot cluster. What has to be taken into account when looking at the results is that Grade D and the APA were analysed separately because the APA can only be entered through the air locks. Furthermore, the recovery rates in Grade D are much higher than in the APA. Because of these facts, adjacent Grade D cleanrooms had to be analysed separately and it correlates with the contamination model from the literature review, as most contaminants can only be brought in through the air locks.

Most factors that influence the results of the hot spot analysis have been taken into consideration and were categorical excluded. The Grade D combined air and surface recovery rate data set is slightly skewed to the right, with an outlier (Grade D corridor – room No. 9 had the highest recovery rate). The APA combined air and surface recovery rate data set has a higher number of 0-values and is also skewed to the right. As a result of the separation of Grade D and the APA, the feature (cleanroom) number was small (29 rooms for Grade D and 25 for the APA), and since the data set is skewed to the right, this may alter the results slightly. Nevertheless, the Grade D hot spot is definitely the sterile corridor in combination with the adjacent air locks, as all main air locks, besides the unisex air lock (rooms No. 97/96) lead to the Grade D corridor (room No. 9) and the contamination route from the GA to Grade D and further

into the APA, with the exception of the unisex air lock (rooms No. 97/96), must go through the sterile corridor. The documenting rooms (rooms No. 17, 16 and 15) that have high air and surface recovery rates are also entered and exited through the Grade D corridor (room No. 9) and so are the equipment washing area (room No. 10) and the vial washing areas in Grade D (rooms No. 29, 31 and 72), which have higher air recovery rates. In addition, the results of the hot spot analysis have to be evaluated in combination with the results of the recovery rate analysis and based on these findings, the conclusion was drawn.

The outcome of the analysis showed that GIS is a great tool to link the data to the geographic environment to provide vital information on the microbiological status of the cleanroom. By pursuing this method of data analysis, the results can be expressed quantitatively and graphically. The obtained results factor different sampling sites within the APA and the rest of the cleanroom, thereby following the approach of ISO 14698-2 (2003). However, this new approach of utilising GIS as an analysis tool reflects the FDA Aseptic Guide, which states that specialised data reports should be established to investigate obtained data beyond established alert and action levels for appropriate actions (Concept Heidelberg, 2004). If the pharmaceutical company changed their environmental monitoring trending programme from a negative binomial distribution calculation regime or log-normal distribution calculation regime to a percentage recovery rate regime, according to the USP Section <1116>, then the establishment of more air sampling points would be required within Grade D. Otherwise the recovery rates would be too high compared with the surface monitoring recovery rates, and this may give the impression that the company does not manufacture according to cGMP in a state of control. As an alternative, the company could run the percentage recovery rate analysis only on the data for cleanroom grades stated in USP section <1116> (A, B, C and perhaps A2 as ISO 6 with a <3% or lower limit) and perform the Grade D EM data trending using the current trending program based on control charting. The control charting program is also established to perform microbiological data trending for water used in pharmaceutical manufacturing and product bioburden. ISO 6 has less stringent non-viable particle limits compared to Grade A2, see Table 1 on page 9 and Table 2 on page 10. Therefore, using the ISO 6 recovery rate limit (<3%) or a lower limit of <2% (which is rounded down half way between the <1% for Grade A and <5% for Grade B) for Grade A2 as viable particle limit for EM data trending may be acceptable.

5.2 Have the air and surface microbial counts changed over time based on 3 years of data?

The data trending from 2009 to 2011 was not uniform in that sense that there were some cleanrooms that were previously air and surface monitored at-rest. This made an otherwise easy graphical comparison harder for the viewer. In addition, the χ^2 calculations had to be adjusted by omitting the 2009 data, which can be seen in Appendix 2: Two-way tables with $\chi 2$ statistics on page 130. Some Grade A2 cleanrooms had no >0 values in the air monitoring data over the three year period of 2009, 2010 and 2011 (portrayed in blue in Figure 18 on page 72), which made a χ^2 calculation impossible and they were therefore depicted in Figure 21 on page 75 as cleanrooms with no statistically significant change in the air monitoring data. The increase in air monitoring RRs in 2011 compared with 2010 was only minor. This was verified by observing the raw data in Appendix 1: Microbiological recovery rate data summary on page 126. Although many cleanrooms (including all air locks) were not monitored in 2009, there was only one cleanroom which had a higher air monitoring RR in 2011 compared with 2009 (Grade A in room No. 21 – filling suite No. 4) while most air monitoring RR of all other cleanrooms have decreased in 2011 compared with 2009. This is contrary to the surface RR data (Figure 24 on page 78) where there were many more cleanrooms that had higher RRs in 2011 compared with 2009.

The results of the χ^2 analysis for the air monitoring data show generally stable air monitoring data. The statistically significant (p-value: <0.05) lower RRs in 2011 compared with 2009/2010 denote a downwards trend in the air monitoring RR data. This is not only positive because continuous improvement is a central statement in Section <1116> of the United States Pharmacopoeia (The United States Pharmacopeial Convention, 2012/a), but also because the air monitoring RR data obtained in Question 1 was significantly higher than its surface monitoring RR counterpart (especially in Grade D). These include such rooms as the documenting rooms, equipment washing and preparation rooms, and the vial washing areas. The results of the χ^2 analysis for the surface monitoring data, on the other hand, show a different picture. Although the statistically significant lower surface RRs observed in 2011 compared with 2009 or 2010 in the documenting rooms (room No. 3, 15, 16 and 17) correlate with the air monitoring RRs, this is not the case for other cleanrooms such as the Grade D men's (room No. 34) and Grade B unisex air lock (room No. 96); the Grade D and Grade B women's air locks (room No. 33 and 23 respectively); part of the APA corridor (room No. 27 and 79); grades A2/A in sterile filling suite No. 1 (room No. 26); Grade A of sterile filling suite No. 2 (room No. 32); and the Grade D vial washing area for sterile filling suite No. 3 (room No. 72). These had statistically significant higher

surface RRs in 2011 compared with 2009 (or 2010 if no monitoring took place in 2009). The surface monitoring RR data set shows a deterioration in the microbiological cleanroom status of the facility between 2009 (or 2010 if no monitoring took place in 2009) and 2011 in these listed cleanrooms, based on the surface monitoring data. However, the downwards trend may also be partially explained by the EM programme, which has improved over the years and has now specific monitoring locations, depicted in SOPs using a grid approach on the floors, walls and ceilings (etc.), in accordance with ISO 14644-2 (2000). This specificity was previously only applied for Grade A and some Grade A2 locations and proves again the importance of a proper environmental monitoring location risk assessment during initial validation and during revalidation. This ensures that all locations – particularly the worst case locations − are monitored uniformly to obtain representative EM results, and also aligns with the EM cleanroom validation and monitoring requirements of ISO 14698-1 (2003). Even so, the statistically significant lower air monitoring RRs correlate by and large with the lower surface monitoring RRs. Similar, this is also the case in the Grade B material air lock (room No. 12) where the statistically significant higher air monitoring RR correlates with the surface RR.

5.3 Is there a certain microbiological flora associated to certain areas of the facility?

The percentage of the microbial classes isolated during the three year period is somewhat similar to the study conducted by Sandle (2011), in that the most frequently isolated class within the APA was G+ve cocci C+ with a percentage of 75.48%, while Sandle reported a recovery of around 81% (although he did not differentiate between C+ and C-). The most predominant G+ve cocci C+ species recovered during EM in this study reflect Cundell's (2004) and Sandle's (2011) findings that human skin bacteria (especially Staphylococci and Micrococci of the same species; *M. luteus*, *S. epidermidis*, and *S. hominis* are the most predominant isolates in pharmaceutical cleanrooms. These findings also correlate with La Duc et al. (2007), who found that 75% of the microorganisms identified were Gram-positive, although 40% of the isolates by La Duc et al. were Bacilli, which is not reflected in this study.

Sandle (2011) reported that 13% of isolates from the APA were G+ve rods S+ while only 3% were G+ve rods S-. This is quite the opposite to the percentage of isolates in this study where only 2.79% of G+ve rods S+ were recovered, while there were 18.59% of G+ve rods S- isolates. Cundel (2004), on the other hand, confirmed the findings of this study when he stated that Corynebacteria spp. are among the most commonly isolated bacteria in aseptic areas. *Corynebacterium spp*. (*Corynebacterium jeikeium* or *Corynebacterium macginleyi*) were among those most commonly isolated in

the G+ve rods S- group. This is of no surprise since they can be found on human skin and/or mucus membranes. Cundel (2004) ranks G+ve rods S+ also on 3rd place of the groups most commonly isolated within cleanrooms. However, while Sandle (2011) reports that *Bacillus sphaericus/ fusiformis* was the most commonly isolated G+ve rods S+ in his research (never isolated during EM in this research), *Bacillus cereus, Bacillus pumilus*, *Bacillus lentus* and *Bacillus firmus* are the most commonly isolated G+ve rods S+ in this study and they correlate with the Bacilli species findings of La Duc et al. (2007). The relatively low recovery of G+ve rods S+ may be an indication of good cleaning/disinfection practices, although the recovery of this class in Grade A is slightly higher compared with the other cleanroom grades. This could be due to the predominantly used disinfectant in Grade A (isopropyl alcohol) not being sporicidal according to the validation. Since G+ve rods S+ produce heat and chemical resistant endospores, it is important to clean/treat the cleanroom surfaces with sporicidal disinfecting agents containing, for example, hypochlorite in addition to the usually used 70% isopropyl alcohol to ensure that these microorganisms are removed and killed off. The sporicidal disinfection in all cleanroom grades is performed monthly unless there is a deviation concerning bacterial endospores or mould spores that requires additional sporicidal cleaning/disinfection.

The 2% of G-ve rods found by Sandle (2011) reflects the 2.04% G-ve rods O+ and 0.02% G-ve rods O-, L+ found in the APA of the study facility in this research. The isolated genera *Sphingomonas spp.* and *Pseudomonas spp*. reflect the findings of the same G-ve rods O+ genera by La Duc et al. (2007). The same goes for the fungi recovery by Sandle (2011) (1%) compared with the recovery in this study (0.89% moulds and 0.04% yeasts). The most commonly isolated mould species in Sandle's study reflects Cundels report and this study in that *Penicillium* and *Aspergillus* are among the moulds most commonly isolated. Although Sandle (2011) used data from 9 years of microbiological EM with around 9000 isolates, the results of his study are a good comparison to this research with 3 years of EM data and 5902 isolates.

The data for this study included EM samples from validation, special investigations and personnel. The validation and special investigation samples are always identified and not just Gram-stained, hence the microbial spectrum is enlarged and this is why these samples have been included in this research. The personnel EM samples have been included to give a more complete picture of the microbiological distribution within the cleanrooms. The incubation temperature for Tryptic Soy Agar (TSA) plates in the study facility is 35°C. The study by Sandle (2011) in comparison used the same parameters (sampling methods, medium etc.) with the exception of the incubation temperature of

25°C for five days after an initial incubation temperature of 35°C for a two day period. This temperature change fosters the growth of moulds and other cooler incubation temperature-requiring microorganisms. However, the Sabourraud Dextrose Agar used in the cleanrooms of the pharmaceutical facility in this study is also incubated at 25°C for at least five days. Hence, some moulds that do not grow on TSA at 35°C will grow on this medium, and would therefore be recovered.

The higher number of moulds in Grade D (20.19%) is probably due to items that are brought into Grade D from the NCA through the material air locks (vials, equipment, etc.) but also personnel air locks (e.g. batch records, pens, hand tools, and other kinds of portable utensils). These contamination sources are stated in ISO 14644-5 (2004), but the high number of moulds may also be due to wet areas (equipment washing areas) and sinks/gullies within Grade D that support viable mould proliferation and not just mould spores. The low number of G-ve rods in Grade D and G-ve rods, O+ in the APA (3.97% in Grade D and 2.04% in the APA) most likely also have their origins in wet areas, since bacteria in this class are often found in water (Sandle, 2011), but they can also be introduced to the cleanrooms by personnel and material, which is most likely the case in the APA, especially since the equipment washing area (room No. 10) is right next to the one chamber Grade B material air lock (room No. 12). The single recovery of a G-ve rods O-, L+ (*Klebsiella pneumoniae ssp. pneumoniae*) is an indication of a high hygiene status, as many bacterial genera of this class belong to the coliform group, which may be of faecal origin. However, *Enterococcus faecalis*, which was isolated in Grade A, is a bacterium from the human intestinal tract, but its habitat can also be in the environment. Hence, the source of its occurrence in Grade A could be attributed to poor hygiene from a staff member, or material movement within the cleanroom, since these microorganisms do also occur in the natural environment (soil, dust, plants, etc.). Furthermore, Cundel (2004) states that this microorganism is infrequently isolated during EM in cleanrooms and therefore not unusual.

The microbial class distribution within the APA cleanroom classes is overall quite similar and differs mainly from Grade D only in the higher number of moulds and a slightly lower numbers of G+ve rods S- in Grade D. It is probably of no surprise that the highest frequency of isolated microbial classes in all cleanroom grades are G+ve cocci C+ followed by G+ve rods S- in the APA. Most microorganisms in this group can be found on the human skin (Cundell, 2004) which can host up to 1×10^6 microorganisms per cm2 (Hall et al., 1986) and this supports the conclusion of Lampe (2013) that personnel is the most important factor and greatest source of microbial contamination within the cleanrooms especially during manual aseptic processing in any

pharmaceutical manufacturing operation. The low number of G+ve cocci C- is an indication of good hygiene practices by the staff members, as many bacteria in this class (e.g. Streptococci) can be shed by humans who may be ill, or who have poor hygiene practices (e.g. Enterococci). The lack of G-ve cocci within the cleanrooms during this 3 year period mirrors the results of Sandle (2011) who did not have any recoveries of this bacterial class in any cleanroom class either.

The findings and conclusion from Sandle (2011) that Grade D isolates show a pattern of far greater variety cannot be confirmed, as the identification of microorganisms in Grade D (and air locks in grades C/B) is only ascertained if the EM sample has an action level excursion. This is also one of the limitations of this study, as the amount of data in Grade D is far less because of the limited identification and due to only monthly EM compared with daily EM in the APA. This is why Sutton (2010), the EU-GMP guideline, ISO 13408-1 and Section <1116> of the USP recommend establishing a programme for regularly identifying microorganisms in grades C and D to obtain a current microbial database (Concept Heidelberg, 2004; ISO 13408-1, 2008; The United States Pharmacopeial Convention, 2012/a). In addition, the EM data generated in Grade A is also much less than in grades A2 and B because of the lower microbial numbers within Grade A, which makes comparisons slightly biased. Nonetheless, the microbial class distribution and genus/species identification for each cleanroom class is quite similar and the bias is therefore not very significant. The findings and conclusion by Sandle (2011) that G-ve rods isolates are more predominant in Grade D compared with the APA can also not be confirmed in this study. Nevertheless, based on the more or less even microbiological genus/species recovery spectrum, it can be concluded that the microorganisms are brought into the cleanrooms through personnel and material flow.

5.4 What is/are the potential contamination route(s) in the cleanrooms?

The potential contamination route findings show that material and personnel flow is responsible for the majority of microbiological contamination of the cleanroom. This correlates with the findings of the literature review. It is therefore essential to control and, if necessary, restrict the movement of personnel and material between cleanrooms, especially those of different grades (Pharmaceutical Inspection Convention, 2009/a). This is why the USP states that sterility assurance is best achieved by focusing on and reducing personnel and other contamination factors through facility and process design (The United States Pharmacopeial Convention, 2012/a).

The developed GIS was a very helpful tool in analysing the EM data and depicting it for easy recognition of patterns. The limitations of this research are that personnel data have only been considered for Question 3 (microbiological flora), since personnel is the biggest contamination factor within the cleanroom environment. The movement of personnel within a cleanroom will always be a limiting variable in any GIS or other analysis. Although there is an entry list to the APA for personnel, it can never be exactly determined which areas of the APA a person has entered. The recovery rate is a great analysis tool but it is less practical in Grade D areas which might be the reason why there is no RR limit in section <1116> of the USP. Another limitation of this research is that some cleanroom regions like the Grade A Air Supply areas have not been considered, as they were not fully implemented during this time period. Nevertheless, it could be shown that this novel approach of utilising a GIS for microbiological data analysis is a great asset to cGMP.

The developed GIS could also be helpful for the EM room validation that requires a risk assessment according to ISO 14698-1 (ISO 14698-1, 2003) and Section <1116> of the United States Pharmacopoeia (The United States Pharmacopeial Convention, 2012/a) for the determination of sampling points. The future of GIS in pharmaceutical manufacturing may also lie in the utilisation of this tool in other areas, such as water monitoring, product bioburden monitoring, and data trending throughout the production process. Finally, the use of a functional GIS fed with microbiological monitoring data can be a useful tool for EM data analysis, CAPA investigations, change control report assessments, and management review reports of product quality and process performance.

6 Conclusions

6.1 Which areas in the manufacturing cleanroom facility have higher air and surface microbial counts?

The Grade D cleanrooms that have the highest combined air and surface recovery rates and hence microbial counts in descending order are the Grade D corridor (room No. 9), which is also the only statistically significant hot spot (p-value: <0.001) in Grade D; the documenting rooms (rooms No. 17, 16, and 15); the vial washing area for sterile filling suite 1 (room No. 29); the men's personnel air lock from the GA to Grade D (room No. 6); the vial washing area for sterile filling suite 2 (room No. 31); the men's personnel air lock from Grade D to Grade B (room No. 34); the personnel air lock from Grade D to Grade B (room No. 97); the vial washing area for sterile filling suite 3 (room No. 72); the equipment washing area for the APA (room No. 10); the women's personnel air lock from Grade D to Grade B (room No. 33); and the equipment preparation area (room No. 18).

The cleanrooms in the APA that have the highest combined air and surface recovery rates and hence microbial counts in descending order are the Grade B men's air lock (room No. 24) and the Grade B women's air lock (room No. 23), which are also statistically significant hot spots in the APA (p-values: <0.001 and 0.02 respectively); the Grade B unisex air lock (room No. 96); the Grade B material air lock (room No. 12); and Grade B in sterile filling suite 4 (room No. 21). The Grade B men's air lock, women's air lock, and unisex air lock also exceed the required recovery rate of <5% by USP section <1116> based on the data of the 3 year period (2009-2011).

6.2 Have the air and surface microbial counts changed over time based on 3 years of data?

The air and surface recovery rates and hence microbial counts in most cleanrooms have remained stable. The air and surface recovery rates in some cleanrooms however have changed over the three year period from 2009 – 2011. The cleanrooms that had statistically significant lower air and surface monitoring recovery rates (p-value: <0.05) in 2011 compared with 2009 (or 2010 if no monitoring in 2009) were the documenting rooms (room No. 17, 16, 15 and 3), the Grade D corridor (room No. 9), and the vial washing room No. 31 for sterile filling suite 2. The only cleanroom that had statistically significant higher air and surface monitoring recovery rates (p-value: <0.05) in 2011 compared with 2010, on the other hand, is the Grade B material air lock (room No. 12). The cleanrooms that had only statistically significant lower air monitoring recovery rates (p-value: <0.05) in 2011 compared with 2009 (or 2010 if no monitoring in 2009) are the equipment washing room (room No. 10), the equipment preparation room (room No.

18), the storage room No. 11, the stopper washing/sterilisation room No. 63, the preparation room No. 81, as well as the vial washing rooms No. 29 and 72 for sterile filling suites 1 and 3 respectively. The only cleanroom that had a statistically significant higher air monitoring recovery rate (p-value: <0.05) in 2011 compared with 2010 was the Grade D men's air lock (room No. 34).

The cleanrooms that had only statistically significant lower surface monitoring recovery rates (p-value: <0.05) in 2011 compared with 2009 were Grade A2 in room 21 (sterile filling suite 4) and Grade A2 in room No. 32 (sterile filling suite 2). The cleanrooms that had statistically significant higher surface monitoring recovery rates (p-value: <0.05) in 2011 compared with 2009 (or 2010 if no monitoring in 2009) were the men's and unisex Grade D air locks (rooms No. 6 and 96 respectively), the Grade D and Grade B women's air locks (rooms No. 33 and 23 respectively), a part of the APA corridor (rooms No. 27 and 79), Grade A2/A in sterile filling suite No. 1 (room No. 26), Grade A of sterile filling suite No. 2 (room No. 32), and the Grade D vial washing area for sterile filling suite No. 3 (room No. 72).

6.3 Is there a certain microbiological flora associated to certain areas of the facility?

The microbial distribution within the APA during the period 2009-2011 is very similar in terms of microbial class distribution and microbial species identification. Grade D has a higher number of moulds (20.19%) and lower number of G+ve rods S- (10.39%) compared with the APA. The Grade D microbial class distribution in descending order is G+ve cocci C+ (61.73%), moulds (20.19%), G+ve rods S- (10.39%), G-ve rods (3.97%), G+ve rods S+ (3.50%), and yeasts (0.23%). The APA microbial class distribution in descending order is G+ve cocci C+ (75.48%), G+ve rods S- (18.59%), G+ve rods S+ (2.79%), G-ve rods O+ (2.04%), moulds (0.89%), G+ve cocci C- (0.14%), yeasts (0.04%), and G-ve rods O-, L+ (0.02%). The identified species in grades A, A2, B and D within each class are almost identical for G+ve cocci C+, G+ve rods S-, G+ve rods S+, and moulds. The identified species in grades A, A2, B, and D within each class are also quite similar for G-ve rods (O+) and yeasts. Finally, there were not many G+ve cocci C- (0.14% within the APA) and only one recovery of a G-ve rods O-, L+ (in Grade B), which is positive, as this group includes coliforms that should not be found in cleanrooms.

6.4 What is/are the potential contamination route(s) in the cleanrooms?

The research shows that the material and personnel flow is responsible for the majority of microbiological contaminations of the cleanrooms, which correlates with the findings of the literature review. Furthermore, the identified additional risks in Table 13 on page

95 contribute to the material and personnel flow contamination routes and hence contamination of the cleanrooms.

7 Recommendations

The following recommendations are made based on the findings in the literature review and due to the research findings, such as those listed in Table 13 on page 95, to improve microbial contamination control within the cleanrooms of the study facility. The equipment used in the APA should be kept in the APA (if possible) in order to minimise the risk of introducing contaminants (ISO 13408-1, 2008). The initial risk assessments in terms of Identification of contamination risks including origins, routes, microbial proliferation, contamination, and adequate removal of microbes should be review and updated. This could require the generation of a microbiological contamination control plan using a quality tool e.g. Hazard Analysis and Critical Control Point (HACCP) plan, etc. (ISO 13408-1, 2008). It should be considered to use a grid approach to disperse EM sampling locations within the cleanrooms during initial cleanroom validation and revalidation (ISO 14644-2, 2000) that takes into account personnel and material movement and critical interventions (The United States Pharmacopeial Convention, 2012/a). The maximum personnel occupancy should be determined and set (ISO 14644-5, 2004) in Grade D (and C) cleanrooms based on a risk assessment/rationale.

A proper exit (undressing) procedure for personnel in air locks should be implemented (ISO 14644-5, 2004) by specifically describing where to put the worn cleanroom gowning items to avoid air lock contamination through placing those items on the floor or bench. This could be resolved by placing bags in the air locks in which the used cleanroom gowning items have to be placed during undressing. The inadequate disposal of waste should be avoided (ISO 14644-5, 2004) in personnel air locks by implementing waste bins in the Grade B personnel air locks. In addition, inadequate aseptic personnel and material flow can be avoided (ISO 14644-5, 2004) through additional training (especially initial training for new staff) and surveillance (reality checks, GMP inspections) by the quality departments against the applicable SOPs. In this regard, simplifying applicable SOPs and providing enough personnel to do the job properly would help in contamination control. Regular reassessments (Pharmaceutical Inspection Convention, 2009/a) and retraining (Lampe, 2013) of aseptic behaviour (e.g. quarterly) in all cleanroom grades ought to be performed, including in grades C and D (e.g. through quality, production and GMP-support departments). In this regard, “train the trainer” courses offered to those staff members in the production department who train others could be considered. Video recording of media-fills and routine fillings for a detailed review of the aseptic behaviour and non-conformance report investigations could also be considered (Lampe, 2013).

Health examinations prior to staff employment and regular re-examinations for all employed personnel who have access to cleanrooms with open product (including quality staff) should be performed (Health Canada, 2009). This could also be implemented (based on a risk assessment) for contractors that are present in these cleanroom areas on a regular basis. Doors must not be left open during air lock cleaning between Grade B and the APA corridor (Grade A2) in order to avoid transferring non-viable and viable particles between rooms (ISO 14644-5, 2004) and to avoid differential pressure alarms. The HVAC capacity should be upgraded to handle seasonal fluctuations in humidity and temperature (Sartain & Polarine, 2011). The use of a cleanroom vacuum cleaner with built in HEPA or ULPA filters could be considered to use on incoming particle shedding materials in the material air lock to reduce particles (ISO 14644-5, 2004). The clean-air inlet in work areas where maintenance is performed could be blocked to achieve a neutral or negative differential pressure (European Commission, 2008; ISO 14644-5, 2004) in order to protect the adjacent cleanrooms from particles. Furthermore, additional EM should implemented around this area of maintenance to detect any contaminants generated by the work performed. The batch record transfer and the transfer of other items between the cleanrooms and the General Area should be kept to a minimum, and pens once brought into the cleanroom should remain there as part of good aseptic practice to avoid cleanroom contamination (ISO 14644-5, 2004). In this respect, substituting conventional paper/pen documentation e.g. batch records with electronic means such as cleanroom-compatible and/or cleanroom-designed computers and tablets can help reducing contamination hazards through avoiding the transfer of office stationaries (batch records, paper, pens, etc.) in and out of the cleanrooms (ISO 14644-5, 2004).

Adjusting the cleaning/disinfection frequency in air locks according to throughput to reduce contaminant transfer to other cleanroom areas and redirecting personnel and material traffic to allow drying of the wet surfaces and any disinfectant to work would help to reduce viable particles (ISO 14644-5, 2004). The reduction and if possible elimination of personnel interventions in Grade A through process design (The United States Pharmacopeial Convention, 2012/a) ought to be pursued in order to avoid contaminating the product. Cleanroom undergarments could be implemented for the APA (worn underneath the cleanroom garment) to reduce particle dispersion by personnel (Sandle, 2011). Conventional cleanroom filling lines are outdated and no longer cGMP conform according to Lysfjord (2012, June) and the FDA. New aseptic filling lines should only be constructed as RABS, or even better isolators (potentially with blow/fill/seal technology where possible), and existing filling lines should be upgraded to RABS, as these technologies are the future of aseptic processing and will

replace conventional cleanroom filling lines. This will result in obtaining Sterility Assurance Levels (SALs) similar to those achieved by terminal sterilisation (Madsen, 2003). Furthermore, The future of conventional aseptic filling of drugs according to Madsen (2003), may lie in the use of sealed suits in the APA equipped with breathing air supply for conventional cleanrooms which could potentially decrease operator-induced contamination in the APA to levels achieved in isolators.

The following additional recommendations are made that do not aid in contamination control, but are merely an improvement in cGMP, since these items are currently not implemented in the study facility. Batch records could be transformed to concise and sound-written aids (Schniepp, 2013) that contain vital information from SOPs, as these are often not at hand during the execution of a task. *Pseudomonas aeruginosa* should be included in the EM and product bioburden objectionable organisms list, as it is defined as an objectionable organism by the British Pharmacopoeia, European Pharmacopoeia and USP (British Pharmacopoeia Commission, 2012; European Directorate for the Quality of Medicines & HealthCare, 2011/b; The United States Pharmacopeial Convention, 2012/c). Growth media used for EM, but also for product bioburden and water bioburden testing should be tested against a selection of microorganisms from an approved culture collection e.g. American Type Culture Collection (ATCC) (The United States Pharmacopeial Convention, 2012/b).
Older microbial cultures may yield Gram-variable results and this is why the USP recommends that individual colonies should be streaked onto fresh media before Gram-staining and further identification (The United States Pharmacopeial Convention, 2012/e). EM should also be performed in the microbiological laboratory to aid an investigation into a possible contamination of microbiological samples within the laboratory environment (Concept Heidelberg, 2004; ISO 13408-1, 2008; The United States Pharmacopeial Convention, 2012/a). A Recovery Rate (RR) trending for EM data could be implemented, as recommended by USP Section <1116> (The United States Pharmacopeial Convention, 2012/a), for grades A-C, but not for Grade D, as there is no RR limit in the USP for Grade D, and because this study showed that this may not be adequate for single sampling point air monitoring data. Moreover, the implementation of a GIS based geographic analysis and support tool could be valuable and improve pharmaceutical cGMP for EM sampling, EM data analysis, CAPA investigations, initial cleanroom validation, change control report assessments, and management review reports of process performance. The GIS tool shall be put into a new user requirement specification as a GMP support tool for a new SQL*LIMS-based software that may replace the older version.

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9 Appendices

9.1 Appendix 1: Microbiological recovery rate data summary

Grade D Air 2009										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	0	0	11	15	0	0	10	11	15	9
>0 Values	0	0	19	15	0	0	164	19	15	21
RR	#DIV/0!	#DIV/0!	63,33	50,00	#DIV/0!	#DIV/0!	94,25	63,33	50,00	70,00
Grade D Air 2010										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	40	18	31	36	16	16	42	34	36	32
>0 Values	6	10	17	12	12	12	134	14	12	16
RR	13,04	35,71	35,42	25,00	42,86	42,86	76,14	29,17	25,00	33,33
Grade D Air 2011										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	54	31	32	35	34	26	41	36	39	31
>0 Values	18	17	16	13	14	22	151	12	9	17
RR	25,00	35,42	33,33	27,08	29,17	45,83	78,65	25,00	18,75	35,42
Grade D Air Total 2009/2010/2011										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	94	49	74	86	50	42	93	81	90	72
>0 Values	24	27	52	40	26	34	449	45	36	54
RR	20,34	35,53	41,27	31,75	34,21	44,74	82,84	35,71	28,57	42,86

Grade D Air 2009										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	10	9	8	13	14	0	0	0	25	0
>0 Values	20	21	22	65	64	0	0	0	29	0
RR	66,67	70,00	73,33	83,33	82,05	#DIV/0!	#DIV/0!	#DIV/0!	53,70	#DIV/0!
Grade D Air 2010										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	27	29	29	43	42	19	22	0	48	0
>0 Values	21	19	19	53	54	9	6	0	24	0
RR	43,75	39,58	39,58	55,21	56,25	32,14	21,43	#DIV/0!	33,33	#DIV/0!
Grade D Air 2011										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	31	28	30	41	39	31	26	0	44	0
>0 Values	17	20	18	55	57	17	22	0	28	0
RR	35,42	41,67	37,50	57,29	59,38	35,42	45,83	#DIV/0!	38,89	#DIV/0!
Grade D Air Total 2009/2010/2011										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	68	66	67	97	95	50	48	0	117	0
>0 Values	58	60	59	173	175	26	28	0	81	0
RR	46,03	47,62	46,83	64,07	64,81	34,21	36,84	#DIV/0!	40,91	#DIV/0!

Grade D Air 2009									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	0	0	0	0	0	0	22	13	0
>0 Values	0	0	0	0	0	0	56	17	0
RR	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	71,79	56,67	#DIV/0!
Grade D Air 2010									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	0	33	54	0	0	0	45	31	14
>0 Values	0	13	34	0	0	0	51	17	14
RR	#DIV/0!	28,26	38,64	#DIV/0!	#DIV/0!	#DIV/0!	53,13	35,42	50,00
Grade D Air 2011									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	0	55	120	0	0	0	47	39	30
>0 Values	0	17	72	0	0	0	49	9	18
RR	#DIV/0!	23,61	37,50	#DIV/0!	#DIV/0!	#DIV/0!	51,04	18,75	37,50
Grade D Air Total 2009/2010/2011									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	0	88	174	0	0	0	114	83	44
>0 Values	0	30	106	0	0	0	156	43	32
RR	#DIV/0!	25,42	37,86	#DIV/0!	#DIV/0!	#DIV/0!	57,78	34,13	42,11

Grade D Surface 2009										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	0	0	82	80	0	0	631	246	266	25
>0 Values	0	0	38	40	0	0	269	54	34	35
RR	#DIV/0!	#DIV/0!	31,67	33,33	#DIV/0!	#DIV/0!	29,89	18,00	11,33	58,33
Grade D Surface 2010										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	148	128	128	93	81	78	645	248	257	29
>0 Values	7	12	12	27	24	27	255	52	43	31
RR	4,52	8,57	8,57	22,50	22,86	25,71	28,33	17,33	14,33	51,67
Grade D Surface 2011										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	329	218	109	90	149	103	689	242	263	47
>0 Values	31	22	11	30	31	77	211	58	37	13
RR	8,61	9,17	9,17	25,00	17,22	42,78	23,44	19,33	12,33	21,67
Grade D Surface Total 2009/2010/2011										
Cleanroom No.	1	2	3	4	5	6	9	10	11	15
0 Values	477	346	319	263	230	181	1965	736	786	101
>0 Values	38	34	61	97	55	104	735	164	114	79
RR	7,38	8,95	16,05	26,94	19,30	36,49	27,22	18,22	12,67	43,89

Grade D Surface 2009										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	32	36	314	433	441	0	0	0	271	0
>0 Values	28	24	46	107	99	0	0	0	29	0
RR	46,67	40,00	12,78	19,81	18,33	#DIV/0!	#DIV/0!	#DIV/0!	9,67	#DIV/0!
Grade D Surface 2010										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	28	28	302	433	471	138	113	20	268	20
>0 Values	32	32	58	107	69	37	62	0	32	0
RR	53,33	53,33	16,11	19,81	12,78	21,14	35,43	0,00	10,67	0,00
Grade D Surface 2011										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	46	44	321	453	444	194	176	46	262	45
>0 Values	14	16	39	87	96	106	134	2	38	3
RR	23,33	26,67	10,83	16,11	17,78	35,33	43,23	4,17	12,67	6,25
Grade D Surface Total 2009/2010/2011										
Cleanroom No.	16	17	18	29	31	33	34	40	63	64
0 Values	106	108	937	1319	1356	332	289	66	801	65
>0 Values	74	72	143	301	264	143	196	2	99	3
RR	41,11	40,00	13,24	18,58	16,30	30,11	40,41	2,94	11,00	4,41

Grade D Surface 2009									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	0	0	0	0	0	0	481	171	0
>0 Values	0	0	0	0	0	0	59	9	0
RR	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	10,93	5,00	#DIV/0!
Grade D Surface 2010									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	20	267	257	0	0	0	489	171	99
>0 Values	0	11	29	0	0	0	51	9	41
RR	0,00	3,96	10,14	#DIV/0!	#DIV/0!	#DIV/0!	9,44	5,00	29,29
Grade D Surface 2011									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	42	625	592	0	0	0	458	162	179
>0 Values	6	35	62	0	0	0	82	18	76
RR	12,50	5,30	9,48	#DIV/0!	#DIV/0!	#DIV/0!	15,19	10,00	29,80
Grade D Surface Total 2009/2010/2011									
Cleanroom No.	65	66	67	68	69	70	72	81	97
0 Values	62	892	849	0	0	0	1428	504	278
>0 Values	6	46	91	0	0	0	192	36	117
RR	8,82	4,90	9,68	#DIV/0!	#DIV/0!	#DIV/0!	11,85	6,67	29,62

Grade B Air 2009						
Cleanroom No.	12	21	23	24	35	96
0 Values	0	312	0	0	0	0
>0 Values	0	20	0	0	0	0
RR	0,00	6,02	0,00	0,00	0,00	0,00
Grade B Air 2010						
Cleanroom No.	12	21	23	24	35	96
0 Values	192	355	167	145	196	176
>0 Values	5	17	28	50	1	20
RR	2,54	4,57	14,36	25,64	0,51	10,20
Grade B Air 2011						
Cleanroom No.	12	21	23	24	35	96
0 Values	286	384	273	260	331	294
>0 Values	48	10	60	73	3	39
RR	14,37	2,54	18,02	21,92	0,90	11,71
Grade B Air Total 2009/2010/2011						
Cleanroom No.	12	21	23	24	35	96
0 Values	478	1051	440	405	527	470
>0 Values	53	47	88	123	4	59
RR	9,98	4,28	16,67	23,30	0,75	11,15

Grade B Surface 2009						
Cleanroom No.	12	21	23	24	35	96
0 Values	0	2423	0	0	0	0
>0 Values	0	35	0	0	0	0
RR	0,00	1,42	0,00	0,00	0,00	0,00
Grade B Surface 2010						
Cleanroom No.	12	21	23	24	35	96
0 Values	1259	2651	1367	1144	1126	1310
>0 Values	6	40	65	289	4	128
RR	0,47	1,49	4,54	20,17	0,35	8,90
Grade B Surface 2011						
Cleanroom No.	12	21	23	24	35	96
0 Values	2093	2638	2283	1937	1913	2173
>0 Values	72	53	182	528	2	287
RR	3,33	1,97	7,38	21,42	0,10	11,67
Grade B Surface Total 2009/2010/2011						
Cleanroom No.	12	21	23	24	35	96
0 Values	3352	7712	3650	3081	3039	3483
>0 Values	78	128	247	817	6	415
RR	2,27	1,63	6,34	20,96	0,20	10,65

Grade A2 Air 2009								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	317	111	143	108	86	145	36	12
>0 Values	4	0	0	0	1	2	0	0
RR	1,25	0,00	0,00	0,00	1,15	1,36	0,00	0,00
Grade A2 Air 2010								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	553	135	129	150	135	168	47	80
>0 Values	5	0	0	0	2	1	0	0
RR	0,90	0,00	0,00	0,00	1,46	0,59	0,00	0,00
Grade A2 Air 2011								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	584	130	152	211	164	175	57	146
>0 Values	7	0	0	0	0	1	0	0
RR	1,18	0,00	0,00	0,00	0,00	0,57	0,00	0,00
Grade A2 Air Total 2009/2010/2011								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	1454	376	424	469	385	488	140	238
>0 Values	16	0	0	0	3	4	0	0
RR	1,09	0,00	0,00	0,00	0,77	0,81	0,00	0,00

Grade A2 Surface 2009								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	5710	3439	7247	4034	3320	3951	1039	822
>0 Values	24	11	43	27	25	13	1	3
RR	0,42	0,32	0,59	0,66	0,75	0,33	0,10	0,36
Grade A2 Surface 2010								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	6461	3917	7705	3768	3498	3952	1227	2221
>0 Values	14	33	70	36	21	23	13	14
RR	0,22	0,84	0,90	0,95	0,60	0,58	1,05	0,63
Grade A2 Surface 2011								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	6809	3969	8356	4780	4052	4170	1450	2933
>0 Values	11	21	98	16	24	30	10	17
RR	0,16	0,53	1,16	0,33	0,59	0,71	0,68	0,58
Grade A2 Surface Total 2009/2010/2011								
Cleanroom No.	21	26	27	32	73	74	79	90
0 Values	18980	11325	23308	12582	10870	12073	3716	5976
>0 Values	49	65	211	79	70	66	24	34
RR	0,26	0,57	0,90	0,62	0,64	0,54	0,64	0,57

Grade A Air 2009				
Cleanroom No.	21	26	32	73
0 Values	904	1015	1390	1014
>0 Values	1	3	3	2
RR	0,11	0,29	0,22	0,20
Grade A Air 2010				
Cleanroom No.	21	26	32	73
0 Values	1006	1204	1427	1174
>0 Values	0	0	4	1
RR	0,00	0,00	0,28	0,09
Grade A Air 2011				
Cleanroom No.	21	26	32	73
0 Values	1029	1430	1984	3808
>0 Values	2	1	3	2
RR	0,19	0,07	0,15	0,05
Grade A Air Total 2009/2010/2011				
Cleanroom No.	21	26	32	73
0 Values	2939	3649	4801	5996
>0 Values	3	4	10	5
RR	0,10	0,11	0,21	0,08

Grade A Surface 2009				
Cleanroom No.	21	26	32	73
0 Values	6283	5233	7554	5287
>0 Values	7	2	2	2
RR	0,11	0,04	0,03	0,04
Grade A Surface 2010				
Cleanroom No.	21	26	32	73
0 Values	8285	8031	8373	6686
>0 Values	6	23	11	5
RR	0,07	0,29	0,13	0,07
Grade A Surface 2011				
Cleanroom No.	21	26	32	73
0 Values	10983	10080	17020	13157
>0 Values	7	14	9	13
RR	0,06	0,14	0,05	0,10
Grade A Surface Total 2009/2010/2011				
Cleanroom No.	21	26	32	73
0 Values	25551	23344	32947	25130
>0 Values	20	39	22	20
RR	0,08	0,17	0,07	0,08

9.2 Appendix 2: Two-way tables with χ^2 statistics

Room No.	1	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	40	54	94
>0 values	0	6	18	24
Total	0	46	72	118
Expected	**2009**	**2010**	**2011**	Total
0 values	0	36,6440678	57,3559322	94
>0 values	0	9,3559322	14,6440678	24
Total	0	46	72	118
Σ (O-E)^2/E	#DIV/0!	0,30734254	0,19635774	
	#DIV/0!	1,20375829	0,7690678	
p value:	0,115557213	**DF:** 1	**Chi-Square:**	2,47652636

Room No.	1	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	148	329	477
>0 values	0	7	31	38
Total	0	155	360	515
Expected	**2009**	**2010**	**2011**	Total
0 values	0	143,563107	333,436893	477
>0 values	0	11,4368932	26,5631068	38
Total	0	155	360	515
Σ (O-E)^2/E	#DIV/0!	0,13712451	0,05903972	
	#DIV/0!	1,72127351	0,74110387	
p value:	0,102995129	**DF:** 1	**Chi-Square:**	2,65854162

Room No.	2	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	18	31	49
>0 values	0	10	17	27
Total	0	28	48	76
Expected	**2009**	**2010**	**2011**	Total
0 values	0	18,0526316	30,9473684	49
>0 valuese	0	9,94736842	17,0526316	27
Total	0	28	48	76
Σ (O-E)^2/E	#DIV/0!	0,00015344	8,9509E-05	
	#DIV/0!	0,00027847	0,00016244	
p value:	0,97913695	**DF:** 1	**Chi-Square:**	0,00068387

Room No.	2	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	128	218	346
>0 values	0	12	22	34
Total	0	140	240	380
Expected	**2009**	**2010**	**2011**	Total
0 values	0	127,473684	218,526316	346
>0 valuese	0	12,5263158	21,4736842	34
Total	0	140	240	380
Σ (O-E)^2/E	#DIV/0!	0,00217306	0,00126762	
	#DIV/0!	0,02211411	0,0128999	
p value:	0,844532938	**DF:** 1	**Chi-Square:**	0,03845469

Room No.	3	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	11	31	31	73
>0 values	19	17	16	52
Total	30	48	47	125
Expected	**2009**	**2010**	**2011**	Total
0 values	17,52	28,032	27,448	73
>0 values	12,48	19,968	19,552	52
Total	30	48	47	125
Σ (O-E)^2/E	2,426392694	0,31424886	0,45965841	
	3,406282051	0,44115705	0,64528969	
p value:	0,021354039	**DF:** 2	**Chi-Square:**	7,69302875

Room No.	3	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	82	128	109	319
>0 values	38	12	11	61
Total	120	140	120	380
Expected	**2009**	**2010**	**2011**	Total
0 values	100,7368421	117,526316	100,736842	319
>0 values	19,26315789	22,4736842	19,2631579	61
Total	120	140	120	380
Σ (O-E)^2/E	3,485013474	0,93339147	0,67780344	
	18,22490653	4,88117836	3,54457866	
p value:	1,27719E-07	**DF:** 2	**Chi-Square:**	31,7468719

Room No.	4	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	15	36	35	86
>0 values	15	12	13	40
Total	30	48	48	126
Expected	**2009**	**2010**	**2011**	Total
0 values	20,47619048	32,7619048	32,7619048	86
>0 values	9,523809524	15,2380952	15,2380952	40
Total	30	48	48	126
Σ (O-E)^2/E	1,464562569	0,3200443	0,15289313	
	3,148809524	0,68809524	0,32872024	
p value:	0,047284984	**DF:** 2	**Chi-Square:**	6,103125

Room No.	4	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	80	93	90	263
>0 values	40	27	30	97
Total	120	120	120	360
Expected	**2009**	**2010**	**2011**	Total
0 values	87,66666667	87,6666667	87,6666667	263
>0 values	32,33333333	32,3333333	32,3333333	97
Total	120	120	120	360
Σ (O-E)^2/E	0,670468948	0,32446134	0,06210393	
	1,817869416	0,87972509	0,16838488	
p value:	0,140646335	**DF:** 2	**Chi-Square:**	3,9230136

Room No.	5	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	0	16	34	50
>0 values	0	12	14	26
Total	0	28	48	76
Expected	2009	2010	2011	Total
0 values	0	18,4210526	31,5789474	50
>0 values	0	9,57894737	16,4210526	26
Total	0	28	48	76
Σ (O-E)^2/E	#DIV/0!	0,31819549	0,18561404	
	#DIV/0!	0,6119144	0,35695007	
p value:	0,224924271	DF: 1	Chi-Square:	1,47267399

Room No.	5	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	0	81	149	230
>0 values	0	24	31	55
Total	0	105	180	285
Expected	2009	2010	2011	Total
0 values	0	84,7368421	145,263158	230
>0 values	0	20,2631579	34,7368421	55
Total	0	105	180	285
Σ (O-E)^2/E	#DIV/0!	0,16479242	0,09612891	
	#DIV/0!	0,68913192	0,40199362	
p value:	0,244920599	DF: 1	Chi-Square:	1,35204687

Room No.	6	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	0	16	26	42
>0 values	0	12	22	34
Total	0	28	48	76
Expected	2009	2010	2011	Total
0 values	0	15,4736842	26,5263158	42
>0 values	0	12,5263158	21,4736842	34
Total	0	28	48	76
Σ (O-E)^2/E	#DIV/0!	0,0179019	0,01044277	
	#DIV/0!	0,02211411	0,0128999	
p value:	0,801264071	DF: 1	Chi-Square:	0,06335868

Room No.	6	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	0	78	103	181
>0 values	0	27	77	104
Total	0	105	180	285
Expected	2009	2010	2011	Total
0 values	0	66,6842105	114,315789	181
>0 values	0	38,3157895	65,6842105	104
Total	0	105	180	285
Σ (O-E)^2/E	#DIV/0!	1,92020106	1,12011728	
	#DIV/0!	3,34188837	1,94943489	
p value:	0,003896044	DF: 1	Chi-Square:	8,3316416

Room No.	9	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	10	42	41	93
>0 values	164	134	151	449
Total	174	176	192	542
Expected	2009	2010	2011	Total
0 values	29,85608856	30,199262	32,9446494	93
>0 values	144,1439114	145,800738	159,055351	449
Total	174	176	192	542
Σ (O-E)^2/E	13,20548913	4,61128545	1,96962705	
	2,73521267	0,95512149	0,40796284	
p value:	6,50884E-06	DF: 2	Chi-Square:	23,8846986

Room No.	9	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	631	645	689	1965
>0 values	269	255	211	735
Total	900	900	900	2700
Expected	2009	2010	2011	Total
0 values	655	655	655	1965
>0 values	245	245	245	735
Total	900	900	900	2700
Σ (O-E)^2/E	0,879389313	0,15267176	1,7648855	
	2,351020408	0,40816327	4,71836735	
p value:	0,005873828	DF: 2	Chi-Square:	10,2744976

Room No.	10	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	11	34	36	81
>0 values	19	14	12	45
Total	30	48	48	126
Expected	2009	2010	2011	Total
0 values	19,28571429	30,8571429	30,8571429	81
>0 values	10,71428571	17,1428571	17,1428571	45
Total	30	48	48	126
Σ (O-E)^2/E	3,55978836	0,32010582	0,85714286	
	6,407619048	0,57619048	1,54285714	
p value:	0,001317721	DF: 2	Chi-Square:	13,2637037

Room No.	10	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	246	248	242	736
>0 values	54	52	58	164
Total	300	300	300	900
Expected	2009	2010	2011	Total
0 values	245,3333333	245,333333	245,333333	736
>0 values	54,66666667	54,6666667	54,6666667	164
Total	300	300	300	900
Σ (O-E)^2/E	0,001811594	0,02898551	0,04528986	
	0,008130081	0,1300813	0,20325203	
p value:	0,81157767	DF: 2	Chi-Square:	0,41755037

Room No.	11	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	15	36	39	90
>0 values	15	12	9	36
Total	30	48	48	126
Expected	**2009**	**2010**	**2011**	Total
0 values	21,42857143	34,2857143	34,2857143	90
>0 values	8,571428571	13,7142857	13,7142857	36
Total	30	48	48	126
Σ (O-E)^2/E	1,928571429	0,08571429	0,64821429	
	4,821428571	0,21428571	1,62053571	
p value:	0,009472381	**DF:** 2	**Chi-Square:**	9,31875

Room No.	11	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	266	257	263	786
>0 values	34	43	37	114
Total	300	300	300	900
Expected	**2009**	**2010**	**2011**	Total
0 values	262	262	262	786
>0 values	38	38	38	114
Total	300	300	300	900
Σ (O-E)^2/E	0,061068702	0,09541985	0,00381679	
	0,421052632	0,65789474	0,02631579	
p value:	0,531110994	**DF:** 2	**Chi-Square:**	1,2655685

Room No.	15	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	9	32	31	72
>0 values	21	16	17	54
Total	30	48	48	126
Expected	**2009**	**2010**	**2011**	Total
0 values	17,1428571	27,4285714	27,4285714	72
>0 values	12,8571429	20,5714286	20,5714286	54
Total	30	48	48	126
Σ (O-E)^2/E	3,86785714	0,76190476	0,46502976	
	5,15714286	1,01587302	0,62003968	
p value:	0,00262172	**DF:** 2	**Chi-Square:**	11,8878472

Room No.	15	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	25	29	47	101
>0 values	35	31	13	79
Total	60	60	60	180
Expected	**2009**	**2010**	**2011**	Total
0 values	33,66666667	33,6666667	33,6666667	101
>0 values	26,33333333	26,3333333	26,3333333	79
Total	60	60	60	180
Σ (O-E)^2/E	2,231023102	0,64686469	5,28052805	
	2,852320675	0,82700422	6,75105485	
p value:	9,19378E-05	**DF:** 2	**Chi-Square:**	18,5887956

Room No.	16	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	10	27	31	68
>0 values	20	21	17	58
Total	30	48	48	126
Expected	**2009**	**2010**	**2011**	Total
0 values	16,19047619	25,9047619	25,9047619	68
>0 values	13,80952381	22,0952381	22,0952381	58
Total	30	48	48	126
Σ (O-E)^2/E	2,366946779	0,04630602	1,00218838	
	2,775041051	0,05428982	1,17497947	
p value:	0,024480565	**DF:** 2	**Chi-Square:**	7,41975152

Room No.	16	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	32	28	46	106
>0 values	28	32	14	74
Total	60	60	60	180
Expected	**2009**	**2010**	**2011**	Total
0 values	35,33333333	35,3333333	35,3333333	106
>0 values	24,66666667	24,6666667	24,6666667	74
Total	60	60	60	180
Σ (O-E)^2/E	0,314465409	1,52201258	3,22012579	
	0,45045045	2,18018018	4,61261261	
p value:	0,002133645	**DF:** 2	**Chi-Square:**	12,299847

Room No.	17	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	9	29	28	66
>0 values	21	19	20	60
Total	30	48	48	126
Expected	**2009**	**2010**	**2011**	Total
0 values	15,71428571	25,1428571	25,1428571	66
>0 values	14,28571429	22,8571429	22,8571429	60
Total	30	48	48	126
Σ (O-E)^2/E	2,868831169	0,59172078	0,32467532	
	3,155714286	0,65089286	0,35714286	
p value:	0,018788907	**DF:** 2	**Chi-Square:**	7,94897727

Room No.	17	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	36	28	44	108
>0 values	24	32	16	72
Total	60	60	60	180
Expected	**2009**	**2010**	**2011**	Total
0 values	36	36	36	108
>0 values	24	24	24	72
Total	60	60	60	180
Σ (O-E)^2/E	0	1,77777778	1,77777778	
	0	2,66666667	2,66666667	
p value:	0,011743628	**DF:** 2	**Chi-Square:**	8,88888889

Room No.	18	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	8	29	30	67
>0 values	22	19	18	59
Total	30	48	48	126
Expected	2009	2010	2011	Total
0 values	15,95238095	25,5238095	25,5238095	67
>0 values	14,04761905	22,4761905	22,4761905	59
Total	30	48	48	126
Σ (O-E)^2/E	3,964321251	0,47343639	0,78500355	
	4,501856336	0,53763115	0,89144471	
p value:	0,00378448	DF: 2	Chi-Square:	11,1536934

Room No.	18	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	314	302	321	937
>0 values	46	58	39	143
Total	360	360	360	1080
Expected	2009	2010	2011	Total
0 values	312,3333333	312,333333	312,333333	937
>0 values	47,66666667	47,6666667	47,6666667	143
Total	360	360	360	1080
Σ (O-E)^2/E	0,008893632	0,34187122	0,24048381	
	0,058275058	2,24009324	1,57575758	
p value:	0,10723986	DF: 2	Chi-Square:	4,46537454

Room No.	29	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	13	43	41	97
>0 values	65	53	55	173
Total	78	96	96	270
Expected	2009	2010	2011	Total
0 values	28,02222222	34,4888889	34,4888889	97
>0 values	49,97777778	61,5111111	61,5111111	173
Total	78	96	96	270
Σ (O-E)^2/E	8,053150057	2,10035796	1,22922394	
	4,515350032	1,17765735	0,68921805	
p value:	0,0001388	DF: 2	Chi-Square:	17,7649574

Room No.	29	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	433	433	453	1319
>0 values	107	107	87	301
Total	540	540	540	1620
Expected	2009	2010	2011	Total
0 values	439,6666667	439,666667	439,666667	1319
>0 values	100,3333333	100,333333	100,333333	301
Total	540	540	540	1620
Σ (O-E)^2/E	0,101086682	0,10108668	0,40434673	
	0,442967885	0,44296788	1,77187154	
p value:	0,1955061	DF: 2	Chi-Square:	3,2643274

Room No.	31	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	14	42	39	95
>0 values	64	54	57	175
Total	78	96	96	270
Expected	2009	2010	2011	Total
0 values	27,44444444	33,7777778	33,7777778	95
>0 values	50,55555556	62,2222222	62,2222222	175
Total	78	96	96	270
Σ (O-E)^2/E	6,586144849	2,00146199	0,80738304	
	3,575335775	1,08650794	0,43829365	
p value:	0,000711907	DF: 2	Chi-Square:	14,4951272

Room No.	31	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	441	471	444	1356
>0 values	99	69	96	264
Total	540	540	540	1620
Expected	2009	2010	2011	Total
0 values	452	452	452	1356
>0 values	88	88	88	264
Total	540	540	540	1620
Σ (O-E)^2/E	0,267699115	0,79867257	0,14159292	
	1,375	4,10227273	0,72727273	
p value:	0,024569363	DF: 2	Chi-Square:	7,41251006

Room No.	33	Air	Grade D	
Observed	2009	2010	2011	Total
0 values	0	19	31	50
>0 values	0	9	17	26
Total	0	28	48	76
Expected	2009	2010	2011	Total
0 values	0	18,4210526	31,5789474	50
>0 values	0	9,57894737	16,4210526	26
Total	0	28	48	76
Σ (O-E)^2/E	#DIV/0!	0,01819549	0,01061404	
	#DIV/0!	0,03499132	0,02041161	
p value:	0,771667962	DF: 1	Chi-Square:	0,08421245

Room No.	33	Surface	Grade D	
Observed	2009	2010	2011	Total
0 values	0	138	194	332
>0 values	0	37	106	143
Total	0	175	300	475
Expected	2009	2010	2011	Total
0 values	0	122,315789	209,684211	332
>0 values	0	52,6842105	90,3157895	143
Total	0	175	300	475
Σ (O-E)^2/E	#DIV/0!	2,01114231	1,17316635	
	#DIV/0!	4,66922551	2,72371488	
p value:	0,001144879	DF: 1	Chi-Square:	10,5772491

Room No.	34	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	22	26	48
>0 values	0	6	22	28
Total	0	28	48	76
Expected	**2009**	**2010**	**2011**	Total
0 values	0	17,6842105	30,3157895	48
>0 values	0	10,3157895	17,6842105	28
Total	0	28	48	76
Σ (O-E)^2/E	#DIV/0!	1,05325815	0,61440058	
	#DIV/0!	1,80558539	1,05325815	
p value:	0,033373763	**DF:** 1	**Chi-Square:**	4,52650227

Room No.	34	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	113	176	289
>0 values	0	62	134	196
Total	0	175	310	485
Expected	**2009**	**2010**	**2011**	Total
0 values	0	104,278351	184,721649	289
>0 values	0	70,7216495	125,278351	196
Total	0	175	310	485
Σ (O-E)^2/E	#DIV/0!	0,72946273	0,41179347	
	#DIV/0!	1,07558534	0,60718528	
p value:	0,092863165	**DF:** 1	**Chi-Square:**	2,82402682

Room No.	63	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	25	48	44	117
>0 values	29	24	28	81
Total	54	72	72	198
Expected	**2009**	**2010**	**2011**	Total
0 values	31,90909091	42,5454545	42,5454545	117
>0 values	22,09090909	29,4545455	29,4545455	81
Total	54	72	72	198
Σ (O-E)^2/E	1,495985496	0,6993007	0,04972805	
	2,160867939	1,01010101	0,07182941	
p value:	0,064318608	**DF:** 2	**Chi-Square:**	5,4878126

Room No.	63	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	271	268	262	801
>0 values	29	32	38	99
Total	300	300	300	900
Expected	**2009**	**2010**	**2011**	Total
0 values	267	267	267	801
>0 values	33	33	33	99
Total	300	300	300	900
Σ (O-E)^2/E	0,059925094	0,00374532	0,09363296	
	0,484848485	0,03030303	0,75757576	
p value:	0,489184617	**DF:** 2	**Chi-Square:**	1,43003064

Room No.	64	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	20	45	65
>0 values	0	0	3	3
Total	0	20	48	68
Expected	**2009**	**2010**	**2011**	Total
0 values	0	19,1176471	45,8823529	65
>0 values	0	0,88235294	2,11764706	3
Total	0	20	48	68
Σ (O-E)^2/E	#DIV/0!	0,04072398	0,01696833	
	#DIV/0!	0,88235294	0,36764706	
p value:	0,2528129	**DF:** 1	**Chi-Square:**	1,30769231

Room No.	65	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	20	42	62
>0 values	0	0	6	6
Total	0	20	48	68
Expected	**2009**	**2010**	**2011**	Total
0 values	0	18,2352941	43,7647059	62
>0 values	0	1,76470588	4,23529412	6
Total	0	20	48	68
Σ (O-E)^2/E	#DIV/0!	0,17077799	0,0711575	
	#DIV/0!	1,76470588	0,73529412	
p value:	0,09774631	**DF:** 1	**Chi-Square:**	2,74193548

Room No.	66	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	33	55	88
>0 values	0	13	17	30
Total	0	46	72	118
Expected	**2009**	**2010**	**2011**	Total
0 values	0	34,3050847	53,6949153	88
>0 values	0	11,6949153	18,3050847	30
Total	0	46	72	118
Σ (O-E)^2/E	#DIV/0!	0,04964996	0,03172081	
	#DIV/0!	0,14563989	0,09304771	
p value:	0,571572567	**DF: 1**	**Chi-Square:**	0,32005837

Room No.	66	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	267	625	892
>0 values	0	11	35	46
Total	0	278	660	938
Expected	**2009**	**2010**	**2011**	Total
0 values	0	264,366738	627,633262	892
>0 values	0	13,6332623	32,3667377	46
Total	0	278	660	938
Σ (O-E)^2/E	#DIV/0!	0,02622898	0,01104796	
	#DIV/0!	0,50861415	0,21423445	
p value:	0,383289237	**DF: 1**	**Chi-Square:**	0,76012554

Room No.	67	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	54	120	174
>0 values	0	34	72	106
Total	0	88	192	280
Expected	**2009**	**2010**	**2011**	Total
0 values	0	54,6857143	119,314286	174
>0 values	0	33,3142857	72,6857143	106
Total	0	88	192	280
Σ (O-E)^2/E	#DIV/0!	0,0085983	0,00394089	
	#DIV/0!	0,01411419	0,006469	
p value:	0,855586229	**DF: 1**	**Chi-Square:**	0,03312238

Room No.	67	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	257	592	849
>0 values	0	29	62	91
Total	0	286	654	940
Expected	**2009**	**2010**	**2011**	Total
0 values	0	258,312766	590,687234	849
>0 values	0	27,687234	63,312766	91
Total	0	286	654	940
Σ (O-E)^2/E	#DIV/0!	0,00667158	0,00291754	
	#DIV/0!	0,06224365	0,0272197	
p value:	0,752969684	**DF: 1**	**Chi-Square:**	0,09905247

Room No.	72	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	22	45	47	114
>0 values	56	51	49	156
Total	78	96	96	270
Expected	**2009**	**2010**	**2011**	Total
0 values	32,93333333	40,5333333	40,5333333	114
>0 values	45,06666667	55,4666667	55,4666667	156
Total	78	96	96	270
Σ (O-E)^2/E	3,629689609	0,49221491	1,0316886	
	2,652465483	0,35969551	0,75392628	
p value:	0,011564211	**DF: 2**	**Chi-Square:**	8,9196804

Room No.	72	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	481	489	458	1428
>0 values	59	51	82	192
Total	540	540	540	1620
Expected	**2009**	**2010**	**2011**	Total
0 values	476	476	476	1428
>0 values	64	64	64	192
Total	540	540	540	1620
Σ (O-E)^2/E	0,052521008	0,35504202	0,68067227	
	0,390625	2,640625	5,0625	
p value:	0,010142785	**DF: 2**	**Chi-Square:**	9,18198529

Room No.	81	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	13	31	39	83
>0 values	17	17	9	43
Total	30	48	48	126
Expected	**2009**	**2010**	**2011**	Total
0 values	19,76190476	31,6190476	31,6190476	83
>0 values	10,23809524	16,3809524	16,3809524	43
Total	30	48	48	126
Σ (O-E)^2/E	2,313711991	0,01211991	1,72296328	
	4,466002215	0,02339424	3,32571982	
p value:	0,002653288	**DF: 2**	**Chi-Square:**	11,8639115

Room No.	81	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	171	171	162	504
>0 values	9	9	18	36
Total	180	180	180	540
Expected	**2009**	**2010**	**2011**	Total
0 values	168	168	168	504
>0 values	12	12	12	36
Total	180	180	180	540
Σ (O-E)^2/E	0,053571429	0,05357143	0,21428571	
	0,75	0,75	3	
p value:	0,089751164	**DF: 2**	**Chi-Square:**	4,82142857

Room No.	97	Air	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	14	30	44
>0 values	0	14	18	32
Total	0	28	48	76
Expected	**2009**	**2010**	**2011**	Total
0 values	0	16,2105263	27,7894737	44
>0 values	0	11,7894737	20,2105263	32
Total	0	28	48	76
Σ (O-E)^2/E	#DIV/0!	0,30143541	0,17583732	
	#DIV/0!	0,41447368	0,24177632	
p value:	0,28702518	**DF:** 2	**Chi-Square:**	1,13352273

Room No.	97	Surface	Grade D	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	99	179	278
>0 values	0	41	76	117
Total	0	140	255	395
Expected	**2009**	**2010**	**2011**	Total
0 values	0	98,5316456	179,468354	278
>0 values	0	41,4683544	75,5316456	117
Total	0	140	255	395
Σ (O-E)^2/E	#DIV/0!	0,00222625	0,00122225	
	#DIV/0!	0,00528972	0,00290416	
p value:	0,914075136	**DF:** 2	**Chi-Square:**	0,01164238

Room No.	12	Air	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	192	286	478
>0 values	0	5	48	53
Total	0	197	334	531
Expected	**2009**	**2010**	**2011**	Total
0 values	0	177,3371	300,6629	478
>0 values	0	19,6629002	33,3370998	53
Total	0	197	334	531
Σ (O-E)^2/E	#DIV/0!	1,21238388	0,7150887	
	#DIV/0!	10,9343301	6,44929052	
p value:	1,11059E-05	**DF:** 1	**Chi-Square:**	19,3110932

Room No.	12	Surface	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	1259	2093	3352
>0 values	0	6	72	78
Total	0	1265	2165	3430
Expected	**2009**	**2010**	**2011**	Total
0 values	0	1236,23324	2115,76676	3352
>0 values	0	28,7667638	49,2332362	78
Total	0	1265	2165	3430
Σ (O-E)^2/E	#DIV/0!	0,41927811	0,24498236	
	#DIV/0!	18,0182081	10,5279599	
p value:	6,49288E-08	**DF:** 1	**Chi-Square:**	29,2104284

Room No.	21	Air	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	312	355	384	1051
>0 values	20	17	10	47
Total	332	372	394	1098
Expected	**2009**	**2010**	**2011**	Total
0 values	317,7887067	356,076503	377,134791	1051
>0 values	14,21129326	15,9234973	16,8652095	47
Total	332	372	394	1098
Σ (O-E)^2/E	0,105444671	0,00325452	0,1249715	
	2,35792233	0,07277661	2,79457549	
p value:	0,065253698	**DF:** 2	**Chi-Square:**	5,45894513

Room No.	21	Surface	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	2423	2651	2638	7712
>0 values	35	40	53	128
Total	2458	2691	2691	7840
Expected	**2009**	**2010**	**2011**	Total
0 values	2417,869388	2647,06531	2647,06531	7712
>0 values	40,13061224	43,9346939	43,9346939	128
Total	2458	2691	2691	7840
Σ (O-E)^2/E	0,010886933	0,00584867	0,03104562	
	0,655937713	0,35238247	1,87049841	
p value:	0,23147118	**DF:** 2	**Chi-Square:**	2,92659981

Room No.	23	Air	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	167	273	440
>0 values	0	28	60	88
Total	0	195	333	528
Expected	**2009**	**2010**	**2011**	Total
0 values	0	162,5	277,5	440
>0 values	0	32,5	55,5	88
Total	0	195	333	528
Σ (O-E)^2/E	#DIV/0!	0,12461538	0,07297297	
	#DIV/0!	0,62307692	0,36486486	
p value:	0,276233037	**DF:** 1	**Chi-Square:**	1,18553015

Room No.	23	Surface	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	1367	2283	3650
>0 values	0	65	182	247
Total	0	1432	2465	3897
Expected	**2009**	**2010**	**2011**	Total
0 values	0	1341,23685	2308,76315	3650
>0 values	0	90,7631511	156,236849	247
Total	0	1432	2465	3897
Σ (O-E)^2/E	#DIV/0!	0,49487155	0,28748724	
	#DIV/0!	7,31287916	4,24829329	
p value:	0,000442517	**DF:** 1	**Chi-Square:**	12,3435312

Room No.	24	Air	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	145	260	405
>0 values	0	50	73	123
Total	0	195	333	528
Expected	**2009**	**2010**	**2011**	Total
0 values	0	149,573864	255,426136	405
>0 values	0	45,4261364	77,5738636	123
Total	0	195	333	528
Σ (O-E)^2/E	#DIV/0!	0,13986554	0,08190324	
	#DIV/0!	0,46053286	0,26968141	
p value:	0,329215044	**DF:** 1	**Chi-Square:**	0,95198304

Room No.	24	Surface	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	1144	1937	3081
>0 values	0	289	528	817
Total	0	1433	2465	3898
Expected	**2009**	**2010**	**2011**	Total
0 values	0	1132,65085	1948,34915	3081
>0 values	0	300,349153	516,650847	817
Total	0	1433	2465	3898
Σ (O-E)^2/E	#DIV/0!	0,11371844	0,06610893	
	#DIV/0!	0,42884517	0,24930431	
p value:	0,354305476	**DF:** 1	**Chi-Square:**	0,85797685

Room No.	35	Air	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	196	331	527
>0 values	0	1	3	4
Total	0	197	334	531
Expected	**2009**	**2010**	**2011**	Total
0 values	0	195,516008	331,483992	527
>0 values	0	1,48399247	2,51600753	4
Total	0	197	334	531
Σ (O-E)^2/E	#DIV/0!	0,00119811	0,00070667	
	#DIV/0!	0,15785034	0,09310334	
p value:	0,615069509	**DF:** 1	**Chi-Square:**	0,25285845

Room No.	35	Surface	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	1126	1913	3039
>0 values	0	4	2	6
Total	0	1130	1915	3045
Expected	**2009**	**2010**	**2011**	Total
0 values	0	1127,7734	1911,2266	3039
>0 values	0	2,22660099	3,77339901	6
Total	0	1130	1915	3045
Σ (O-E)^2/E	#DIV/0!	0,00278863	0,00164551	
	#DIV/0!	1,41244169	0,83345123	
p value:	0,133586165	**DF:** 1	**Chi-Square:**	2,25032707

Room No.	96	Air	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	176	294	470
>0 values	0	20	39	59
Total	0	196	333	529
Expected	**2009**	**2010**	**2011**	Total
0 values	0	174,139887	295,860113	470
>0 values	0	21,8601134	37,1398866	59
Total	0	196	333	529
Σ (O-E)^2/E	#DIV/0!	0,01986921	0,01169479	
	#DIV/0!	0,15828015	0,09316189	
p value:	0,594737691	**DF:** 2	**Chi-Square:**	0,28300603

Room No.	96	Surface	Grade B	
Observed	**2009**	**2010**	**2011**	Total
0 values	0	1310	2173	3483
>0 values	0	128	287	415
Total	0	1438	2460	3898
Expected	**2009**	**2010**	**2011**	Total
0 values	0	1284,90354	2198,09646	3483
>0 values	0	153,09646	261,90354	415
Total	0	1438	2460	3898
Σ (O-E)^2/E	#DIV/0!	0,49017866	0,28653533	
	#DIV/0!	4,11395725	2,40482542	
p value:	0,006912766	**DF:** 2	**Chi-Square:**	7,29549665

Room No.	21	Air	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	317	553	584	1454
>0 values	4	5	7	16
Total	321	558	591	1470
Expected	**2009**	**2010**	**2011**	Total
0 values	317,5061224	551,926531	584,567347	1454
>0 values	3,493877551	6,07346939	6,43265306	16
Total	321	558	591	1470
Σ (O-E)^2/E	0,000806787	0,00208784	0,00055063	
	0,073316803	0,18973283	0,05003885	
p value:	0,853621943	**DF:** 2	**Chi-Square:**	0,31653375

Room No.	21	Surface	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	5710	6461	6809	18980
>0 values	24	14	11	49
Total	5734	6475	6820	19029
Expected	**2009**	**2010**	**2011**	Total
0 values	5719,234852	6458,32676	6802,43838	18980
>0 values	14,76514793	16,6732356	17,5616165	49
Total	5734	6475	6820	19029
Σ (O-E)^2/E	0,014911521	0,00110651	0,00632932	
	5,775932155	0,42860238	2,45164281	
p value:	0,013046148	**DF:** 2	**Chi-Square:**	8,6785247

Room No.	26	Air	Grade A2	
Observed	2009	2010	2011	Total
0 values	111	135	130	376
>0 values	0	0	0	0
Total	111	135	130	376
Expected	2009	2010	2011	Total
0 values	111	135	130	376
>0 values	0	0	0	0
Total	111	135	130	376
Σ (O-E)^2/E	0	0	0	
	#DIV/0!	#DIV/0!	#DIV/0!	
p value:	#DIV/0!	DF: 2	Chi-Square:	#DIV/0!

Room No.	26	Surface	Grade A2	
Observed	2009	2010	2011	Total
0 values	3439	3917	3969	11325
>0 values	11	33	21	65
Total	3450	3950	3990	11390
Expected	2009	2010	2011	Total
0 values	3430,311677	3927,4583	3967,23003	11325
>0 values	19,68832309	22,5417032	22,7699737	65
Total	3450	3950	3990	11390
Σ (O-E)^2/E	0,02200586	0,02784905	0,00078967	
	3,834097895	4,85216089	0,137585	
p value:	0,011828491	DF: 2	Chi-Square:	8,87448836

Room No.	27	Air	Grade A2	
Observed	2009	2010	2011	Total
0 values	143	129	152	424
>0 values	0	0	0	0
Total	143	129	152	424
Expected	2009	2010	2011	Total
0 values	143	129	152	424
>0 values	0	0	0	0
Total	143	129	152	424
Σ (O-E)^2/E	0	0	0	
	#DIV/0!	#DIV/0!	#DIV/0!	
p value:	#DIV/0!	DF: 2	Chi-Square:	#DIV/0!

Room No.	27	Surface	Grade A2	
Observed	2009	2010	2011	Total
0 values	7247	7705	8356	23308
>0 values	430	70	98	598
Total	7677	7775	8454	23906
Expected	2009	2010	2011	Total
0 values	7484,962604	7580,51117	8242,52623	23308
>0 values	192,0373965	194,488831	211,473772	598
Total	7677	7775	8454	23906
Σ (O-E)^2/E	7,56532847	2,04438312	1,56217847	
	294,8706956	79,68308	60,8883875	
p value:	1,0447E-97	DF: 2	Chi-Square:	446,614053

Room No.	32	Air	Grade A2	
Observed	2009	2010	2011	Total
0 values	108	150	2011	2269
>0 values	0	0	0	0
Total	108	150	2011	2269
Expected	2009	2010	2011	Total
0 values	108	150	2011	2269
>0 values	0	0	0	0
Total	108	150	2011	2269
Σ (O-E)^2/E	0	0	0	
	#DIV/0!	#DIV/0!	#DIV/0!	
p value:	#DIV/0!	DF: 2	Chi-Square:	#DIV/0!

Room No.	32	Surface	Grade A2	
Observed	2009	2010	2011	Total
0 values	4034	3768	4780	12582
>0 values	27	36	16	79
Total	4061	3804	4796	12661
Expected	2009	2010	2011	Total
0 values	4035,660848	3780,26443	4766,07472	12582
>0 values	25,33915173	23,7355659	29,9252824	79
Total	4061	3804	4796	12661
Σ (O-E)^2/E	0,000683511	0,0397899	0,0406862	
	0,108859879	6,33717115	6,47992178	
p value:	0,001498102	DF: 2	Chi-Square:	13,0071124

Room No.	73	Air	Grade A2	
Observed	2009	2010	2011	Total
0 values	86	135	164	385
>0 values	1	2	0	3
Total	87	137	164	388
Expected	2009	2010	2011	Total
0 values	86,32731959	135,940722	162,731959	385
>0 values	0,672680412	1,05927835	1,26804124	3
Total	87	137	164	388
Σ (O-E)^2/E	0,001241068	0,00650988	0,00988084	
	0,159270451	0,83543407	1,26804124	
p value:	0,319758655	DF: 2	Chi-Square:	2,28037754

Room No.	73	Surface	Grade A2	
Observed	2009	2010	2011	Total
0 values	3320	3498	4052	10870
>0 values	25	21	24	70
Total	3345	3519	4076	10940
Expected	2009	2010	2011	Total
0 values	3323,596892	3496,48355	4049,91956	10870
>0 values	21,40310786	22,5164534	26,0804388	70
Total	3345	3519	4076	10940
Σ (O-E)^2/E	0,00389266	0,0006577	0,00106872	
	0,604474506	0,10213113	0,16595677	
p value:	0,644622282	DF: 2	Chi-Square:	0,87818149

Room No.	74	Air	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	145	168	175	488
>0 values	2	1	1	4
Total	147	169	176	492
Expected	**2009**	**2010**	**2011**	Total
0 values	145,804878	167,626016	174,569106	488
>0 values	1,195121951	1,37398374	1,43089431	4
Total	147	169	176	492
Σ (O-E)^2/E	0,004443121	0,00083438	0,00106359	
	0,542060727	0,10179439	0,12975795	
p value:	0,677072395	**DF:** 2	**Chi-Square:**	0,77995415

Room No.	74	Surface	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	3951	3952	4170	12073
>0 values	13	23	30	66
Total	3964	3975	4200	12139
Expected	**2009**	**2010**	**2011**	Total
0 values	3942,447648	3953,38784	4177,16451	12073
>0 values	21,55235192	21,6121592	22,8354889	66
Total	3964	3975	4200	12139
Σ (O-E)^2/E	0,018552618	0,0004872	0,01228829	
	3,39372351	0,08912123	2,24782658	
p value:	0,056078672	**DF:** 2	**Chi-Square:**	5,76199944

Room No.	79	Air	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	36	47	57	140
>0 values	0	0	0	0
Total	36	47	57	140
Expected	**2009**	**2010**	**2011**	Total
0 values	36	47	57	140
>0 values	0	0	0	0
Total	36	47	57	140
Σ (O-E)^2/E	0	0	0	
	#DIV/0!	#DIV/0!	#DIV/0!	
p value:	#DIV/0!	**DF:** 2	**Chi-Square:**	#DIV/0!

Room No.	79	Surface	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	1039	1227	1450	3716
>0 values	1	13	10	24
Total	1040	1240	1460	3740
Expected	**2009**	**2010**	**2011**	Total
0 values	1033,326203	1232,04278	1450,63102	3716
>0 values	6,673796791	7,95721925	9,36898396	24
Total	1040	1240	1460	3740
Σ (O-E)^2/E	0,031153734	0,02064022	0,00027449	
	4,823636535	3,19579452	0,04249994	
p value:	0,017300849	**DF:** 2	**Chi-Square:**	8,11399944

Room No.	90	Air	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	12	80	146	238
>0 values	0	0	0	0
Total	12	80	146	238
Expected	**2009**	**2010**	**2011**	Total
0 values	12	80	146	238
>0 values	0	0	0	0
Total	12	80	146	238
Σ (O-E)^2/E	0	0	0	
	#DIV/0!	#DIV/0!	#DIV/0!	
p value:	#DIV/0!	**DF:** 2	**Chi-Square:**	#DIV/0!

Room No.	90	Surface	Grade A2	
Observed	**2009**	**2010**	**2011**	Total
0 values	822	2221	2933	5976
>0 values	3	14	17	34
Total	825	2235	2950	6010
Expected	**2009**	**2010**	**2011**	Total
0 values	820,3327787	2222,35607	2933,31115	5976
>0 values	4,667221298	12,6439268	16,6888519	34
Total	825	2235	2950	6010
Σ (O-E)^2/E	0,003388414	0,00082747	3,3005E-05	
	0,595563544	0,14544015	0,00580107	
p value:	0,686927295	**DF:** 2	**Chi-Square:**	0,75105364

Room No.	21	Air	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	904	1006	1029	2939
>0 values	1	0	2	3
Total	905	1006	1031	2942
Expected	**2009**	**2010**	**2011**	Total
0 values	904,0771584	1004,97417	1029,94867	2939
>0 values	0,922841604	1,02583277	1,05132563	3
Total	905	1006	1031	2942
Σ (O-E)^2/E	6,58508E-06	0,00104712	0,00087381	
	0,006451181	1,02583277	0,85604597	
p value:	0,388629544	**DF:** 2	**Chi-Square:**	1,89025744

Room No.	21	Surface	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	6382	8285	10983	25650
>0 values	7	6	7	20
Total	6389	8291	10990	25670
Expected	**2009**	**2010**	**2011**	Total
0 values	6384,022205	8284,54032	10981,4375	25650
>0 values	4,977795092	6,45968056	8,56252435	20
Total	6389	8291	10990	25670
Σ (O-E)^2/E	0,000640554	2,5506E-05	0,00022233	
	0,821510853	0,03271156	0,28513581	
p value:	0,56545571	**DF:** 2	**Chi-Square:**	1,14024661

Room No.	26	Air	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	1015	1204	1430	3649
>0 values	3	0	1	4
Total	1018	1204	1431	3653
Expected	**2009**	**2010**	**2011**	Total
0 values	1016,8853	1202,68163	1429,43307	3649
>0 values	1,114700246	1,31836846	1,56693129	4
Total	1018	1204	1431	3653
Σ (O-E)^2/E	0,003495335	0,00144518	0,00022485	
	3,188619696	1,31836846	0,20512137	
p value:	0,094548963	**DF:** 2	**Chi-Square:**	4,7172749

Room No.	26	Surface	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	5233	8031	10080	23344
>0 values	2	23	14	39
Total	5235	8054	10094	23383
Expected	**2009**	**2010**	**2011**	Total
0 values	5226,268657	8040,56691	10077,1644	23344
>0 values	8,731343284	13,4330924	16,8355643	39
Total	5235	8054	10094	23383
Σ (O-E)^2/E	0,008669853	0,01138299	0,00079789	
	5,189462942	6,81345128	0,47758571	
p value:	0,001929151	**DF:** 2	**Chi-Square:**	12,5013507

Room No.	32	Air	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	1390	1427	1984	4801
>0 values	3	4	3	10
Total	1393	1431	1987	4811
Expected	**2009**	**2010**	**2011**	Total
0 values	1390,104552	1428,02557	1982,86988	4801
>0 values	2,895447932	2,97443359	4,13011848	10
Total	1393	1431	1987	4811
Σ (O-E)^2/E	7,86353E-06	0,00073653	0,0006441	
	0,003775283	0,35360899	0,30923272	
p value:	0,716051816	**DF:** 2	**Chi-Square:**	0,66800549

Room No.	32	Surface	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	7554	8373	17020	32947
>0 values	2	11	9	22
Total	7556	8384	17029	32969
Expected	**2009**	**2010**	**2011**	Total
0 values	7550,95793	8378,40541	17017,6367	32947
>0 values	5,042069823	5,59458886	11,3633413	22
Total	7556	8384	17029	32969
Σ (O-E)^2/E	0,001225565	0,00348735	0,00032821	
	1,835394815	5,22263036	0,4915264	
p value:	0,022884479	**DF:** 2	**Chi-Square:**	7,5545927

Room No.	73	Air	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	1014	1174	3808	5996
>0 values	2	1	2	5
Total	1016	1175	3810	6001
Expected	**2009**	**2010**	**2011**	Total
0 values	1015,153474	1174,021	3806,82553	5996
>0 values	0,846525579	0,9790035	3,17447092	5
Total	1016	1175	3810	6001
Σ (O-E)^2/E	0,001310642	3,7551E-07	0,00036234	
	1,571722429	0,00045031	0,43452341	
p value:	0,366343172	**DF:** 2	**Chi-Square:**	2,00836951

Room No.	73	Surface	Grade A	
Observed	**2009**	**2010**	**2011**	Total
0 values	5287	6686	13157	25130
>0 values	2	5	13	20
Total	5289	6691	13170	25150
Expected	**2009**	**2010**	**2011**	Total
0 values	5284,794036	6685,67913	13159,5268	25130
>0 values	4,205964215	5,32087475	10,473161	20
Total	5289	6691	13170	25150
Σ (O-E)^2/E	0,000920808	1,54E-05	0,00048519	
	1,156994655	0,01935032	0,60964547	
p value:	0,409136712	**DF:** 2	**Chi-Square:**	1,78741184

9.3 Appendix 3: Microbial class distribution data in numbers and percent

Microbial class numbers									
Cleanroom grade	**Moulds**	**Yeasts**	**G+ve cocci C+**	**G+ve cocci C-**	**G+ve rods S-**	**G+ve rods S+**	**G-ve rods O+**	**G-ve rods O- L+**	**Sum**
D	173	2	529	0	89	30	34	0	857
B	27	1	2080	0	508	38	47	1	2702
A2	13	0	1549	3	386	74	42	0	2067
A	5	1	179	4	44	29	14	0	276

Microbial class percent									
Cleanroom grade	**Moulds**	**Yeasts**	**G+ve cocci C+**	**G+ve cocci C-**	**G+ve rods S-**	**G+ve rods S+**	**G-ve rods O+**	**G-ve rods O- L+**	**Sum %**
D	20,19	0,23	61,73	0,00	10,39	3,50	3,97	0,00	100,00
B	1,00	0,04	76,98	0,00	18,80	1,41	1,74	0,04	100,00
A2	0,63	0,00	74,94	0,15	18,67	3,58	2,03	0,00	100,00
A	1,81	0,36	64,86	1,45	15,94	10,51	5,07	0,00	100,00

Microbial class numbers									
Cleanroom grade	**Moulds**	**Yeasts**	**G+ve cocci C+**	**G+ve cocci C-**	**G+ve rods S-**	**G+ve rods S+**	**G-ve rods O+**	**G-ve rods O- L+**	**Sum**
APA	45	2	3808	7	938	141	103	1	5045

Microbial class percent									
Cleanroom grade	**Moulds**	**Yeasts**	**G+ve cocci C+**	**G+ve cocci C-**	**G+ve rods S-**	**G+ve rods S+**	**G-ve rods O+**	**G-ve rods O- L+**	**Sum %**
APA	0,89	0,04	75,48	0,14	18,59	2,79	2,04	0,02	100,00