Universitat Autònoma de Barcelona

Development and application of analytical techniques for quality control of biologics and sterile pharmaceutical products

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Este proyecto de investigación se ha elaborado en colaboración con la empresa Laboratorios Reig Jofré en el marco del Plan de Doctorados Industriales, convocatoria DI 2015.

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Dra. Elisabet Rosell i Vives

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List of Abbreviations and Acronyms

FDA: Food and Drug Administration

RJ: Laboratorios Reig Jofré

OTC: over-the-counter

ENT: ear, nose and throat

HEPA: High-Efficiency Particulate Arrestor

ISO: International Standard Organization

IO: In Operation

AR: At Rest

GMP: Good Manufacturing Practices

MHRA: Medicines and Health Care Products Regulatory Agency

PMDA: Pharmaceutical and Medical Devices Agency in Japan

PIC/S: Pharmaceutical Inspection and Co-operation Scheme (PIC/)

WHO: World Health Organisation

CFR: Code of Federal Regulations

EU: European Union

USA: United States of America

EP: European Pharmacopoeia

USP: United States Pharmacopoeia

PDA: Parenteral Drug Association

TR: Technical Report

IPC: In Process Control

QC: Quality Control

QA: Quality Assurance

ICH: International Conference Harmonization

QbT: Quality by Testing

QbD: Quality by Design

QbR: Question-based Review

CMC: Chemical Manufacturing Controls

ANDA: Abbreviated New Drug Application

PAT: Process Analytical Technology

API: Active Pharmaceutical Ingredients

CFU: Colony Forming Unit

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

TVC: Total Viable Count

RMM: Rapid Microbiological Methods

EMA: European Medicines Agency

MALDI-TOF: Mass-Assisted Laser-Detection Ionization Time-of-Flight

PCR: Polymerase Chain Reaction

CO_2: Carbon Dioxide

LED: Light Emitting Diode

CCD: Charged Coupled Device

ATP: Adenosine Triphosphate

ADP: Adenosine Diphosphate

RLU: Relative Light Unit

NADH: Nicotine-adenine dihydroxynucleotide

PMS: Particle Measuring Systems

Ms: millisecond

UV: Ultra-Violet

PMT: Photomultiplier Tube

GC: Gas Chromatography

IVD: In-Vitro Diagnostics

m/z: mass/charge

rRNA: ribosomal Ribonucleic Acid

DNA: Deoxyribonucleic Acid

NCBI: National Center of Biotechnology Information

SSU: Small Subunit

LSU: Large Subunit

ITS: Internal Transcribed Spacer

NDA: New Drug Application

ATCC: American Type Culture Collection

R2A: Reasoner's 2A Agar

PDA: Potato Dextrose Agar

CHCA: α-cyano-4-hydroxycinnamic

Hz: Hertzs

Kv: Kilovolts

Da: Daltons

TE: Tris-EDTA

EDTA: Ethylenediamine tetraacetic acid

M: Molar

mM: Milimolar

BLAST: Basic Local Alignment Search Tool

RefSeq: Reference Sequence Database

PBS: Phosphate Buffer Solution

WFI: Water for Injection

IQ: Installation Qualification

OQ: Operational Qualification

PQ: Performance Qualification

IL: Inferior Limit (Alert Level)

SL: Superior Limit (Action Level)

URS: User Requirements Specification

MRSA: Methicilin-Resistance Staphylococcus Aureus

IMD: Instantaneous Microbial Detection

VBNC: Viable but non-culturable

CAPA: Corrective actions and preventive actions

ROI: Return of Investment

Content Summary

Microbiological testing plays an essential role in the Pharmaceutical Industry as it is an indicator of safety in drug products. The microbiological methods used in the pharmaceutical companies for testing the environment of the manufacturing process as well as the final products are based on traditional culture methods. These methods, although being appropriate for their intended use, rely on century-old technology that lacks accuracy when compared to most up-to-date methodologies for microbial detection and identification.

Every time the requirement of microbiological testing of products and processes increases, arising the need of faster and more accurate methods. In this paradigm, rapid microbiological methods (RMM) are developed for their implementation in the pharmaceutical industry encouraged by regulatory agencies. The different technologies in which rapid microbiological methods are based have been known in the academic field for decades, however their implementation and validation in the pharmaceutical industries is relatively recent.

Implementation of new methodologies in a pharmaceutical environment ruled under Good Manufacturing Practice (GMP) guidelines needs proper validation of the techniques involved. This thesis describes the implementation of RMM in the microbial monitoring of the pharmaceutical manufacturing process and products in Reig Jofré Laboratories (RJF). The main objective has been divided into three different blocks: implementation of a microbial identification program for the isolates found in the production process and products; implementation of a laser-induced fluorescence system for the detection of airborne microorganisms in cleanrooms for aseptic processing and evaluation of a solid-phase cytometry system for the detection of microorganisms in filterable products.

In general, the RMM implemented have contributed to obtain faster results which allows to mitigate contamination risk at the moment it is detected. This projects lays the ground for further applications of RMM in the pharmaceutical manufacturing process.

Objectives

Reig Jofré, as a pharmaceutical company focused in the manufacturing of sterile products, has the initiative to apply innovative microbiological control techniques that improve aseptic manufacturing. For that reason, the main objective of this thesis is the implementation of rapid microbiological methods in the pharmaceutical manufacturing process and the testing of final products.

This global objective has been approached by defining specific objectives:

1. Firstly, a microbial identification program that covered the requirements of GMP guidelines was established and implemented in the company:
    a. For that, microbial identification methods that fitted the company needs have been chosen and validated according to cGMP.
    b. Once validated, microbial identification methods were implemented in routine and their performance was evaluated
2. The second objective was to implement a nearly-real-time method based in laser-induced fluorescence for the detection of viable particles in cleanroom air. This objective implied:
    a. The qualification of the system for operation in classified areas according to cGMP.
    b. Setting new alert and action limits in base to internal data obtained with the already qualified system.
    c. Evaluation of the system performance during a defined time period.
3. The third objective was to evaluate a rapid detection system based on solid-phase cytometry for the detection of microorganisms in water of pharmaceutical use and other filterable products. For that, different tests were carried out:
    a. Filterability of internal water samples was assessed and the alternative system was compared with the traditional one.
    b. Two different products were assayed for possible fluorescent interferes.

Introduction

1 Introduction to the Pharmaceutical Industry

Pharmaceutical industry is formed by activities that comprise research, development, manufacturing and distribution of drugs and devices for human or veterinary use. These activities are carried out by a large diversity of companies that compromise with the quality standards required by drugs as products directly related with human an animal health.

According to Food and Drug Administration (FDA) the term *drug* has different definitions, one of which is a substance with intended use for diagnosis, cure, mitigation, treatment or prevention of disease as well as a substance (other than food) intended to affect any structure of the body[1].

2 Laboratorios Reig Jofre

The project described in this dissertation has been an initiative of Laboratorios Reig Jofré (RJF). In brief, RJF is a Spanish pharmaceutical company founded in 1929. RJF major activities are research, development, manufacturing and marketing of pharmaceutical products and nutritional supplements, as well as on specialized contract development and manufacturing.

The company directs its activity in two business units:

• **Technological specialization area** (49.7% of total sales) in the development and manufacture of antibiotics derived from penicillin and sterile injectable products, which made possible to grow internationally and distribute or license Reig Jofre products in more than 65 countries on five continents.

• **Therapeutic areas and health care** (50.3% of sales) focused on the research, development, manufacture and marketing of our own developments in dermatology and gynaecology, mainly, and in the development of over-the-counter (OTC) products in the field of ear, nose and throat (ENT) and topical disinfectants. This area also includes the line of nutritional supplements, marketed under **Forté Pharma** brand, mainly in France, Belgium, Spain and Portugal.

Reig Jofre has about 1,000 employees, 4 development and manufacturing centres in Europe (Spain: 1 in Barcelona &, 2 in Toledo; and 1 in Sweden), direct sales in 7 countries and over 130 commercial partners in more than 65 countries worldwide[2].

Being sterile injectable products one of the main pillars of the company, development and application of innovational techniques for improvement of aseptic manufacturing facilities is of great interest. For that, the company has taken the initiative to implement Rapid Microbiological Methods for microbial monitoring as they are an improvement in the safety and quality of processes and products.

3 Types of products in the Pharmaceutical Industry and insight on sterile products

Drug products can be classified according to its chemical structure, therapeutic function, dosage form or route of administration, among others. However, what determines the manufacturing process and its regulation is the characteristic if the product is sterile or not.

Parenteral drug delivery systems and many medicinal products such as dressings and sutures must be sterile in order to avoid the possibilities of microbial degradation or

infection occurring as a result of their use[3]. A common general classification of pharmaceutical products is shown in figure 1.

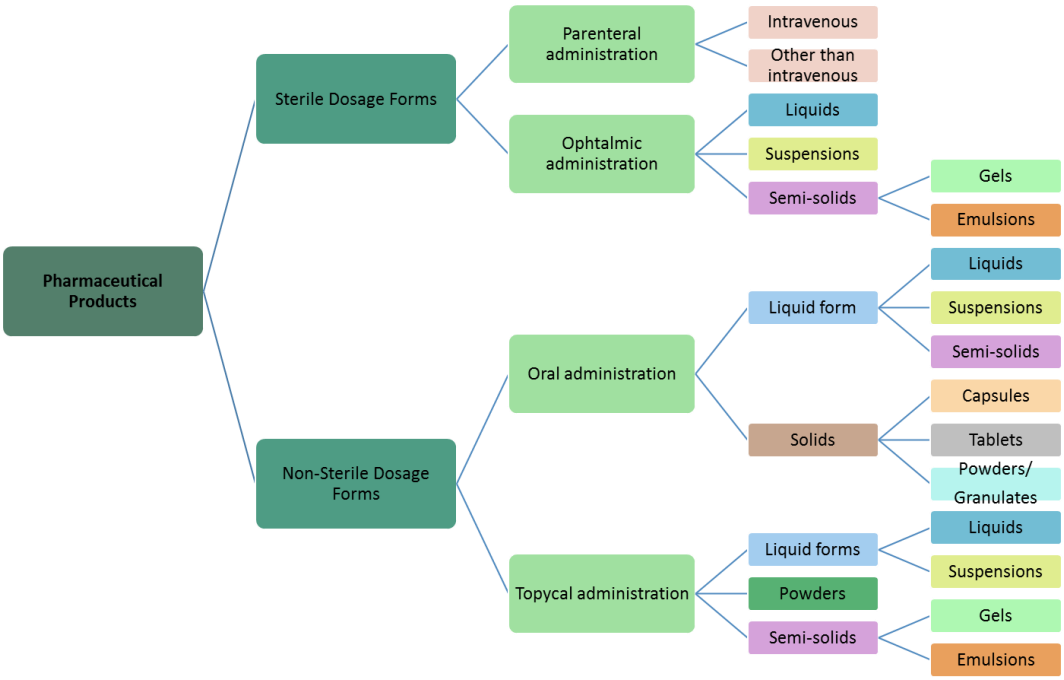

Figure 1. Types of pharmaceutical products classified into sterile and non-sterile dosage forms

Pharmaceuticals can be classified according to body administration routes, for example, topical and oral or parenteral. Pharmaceuticals in contact with vulnerable areas of the body, such as intravenous injections or ophthalmic preparations, are manufactured as sterile, while products administered orally or in superficial skin are not necessarily sterile, although they must accomplish certain microbial content specifications.

4 Sterile pharmaceutical manufacturing process

All activities taking part in the pharmaceutical manufacturing processes are complicated as they must meet high quality standards and specifications, ensuring a total control over the process, so that, this is achieved for every dosage form. In addition, sterile manufacturing needs to ensure that all dosage forms are free from microbial contamination, endotoxin contamination and particulate matter. For this, not only sterile manufacturing facilities need mechanical excellence but they require absolute cleanliness, sanitation and sterilization of all product-contact components[4,5].

A sterile pharmaceutical manufacturing process involves different unit operations such as compounding and mixing, filtration, filling, terminal sterilization (when possible), freeze-drying (lyophilization) if needed, closing and sealing, sorting and inspection, labeling and final packaging for distribution[5].

Figure 2 shows a schematic representation of the pharmaceutical manufacturing process. The process starts with procurement of approved raw materials (active ingredients, excipients, vehicles, etc.) and primary packaging components (containers, closures, etc.) and ending with the final product completely sterile sealed in its dispending package. Every step in the process must be controlled very carefully so the product will have its required quality, regarding its composition and effectiveness as well as sterility characteristic[5].

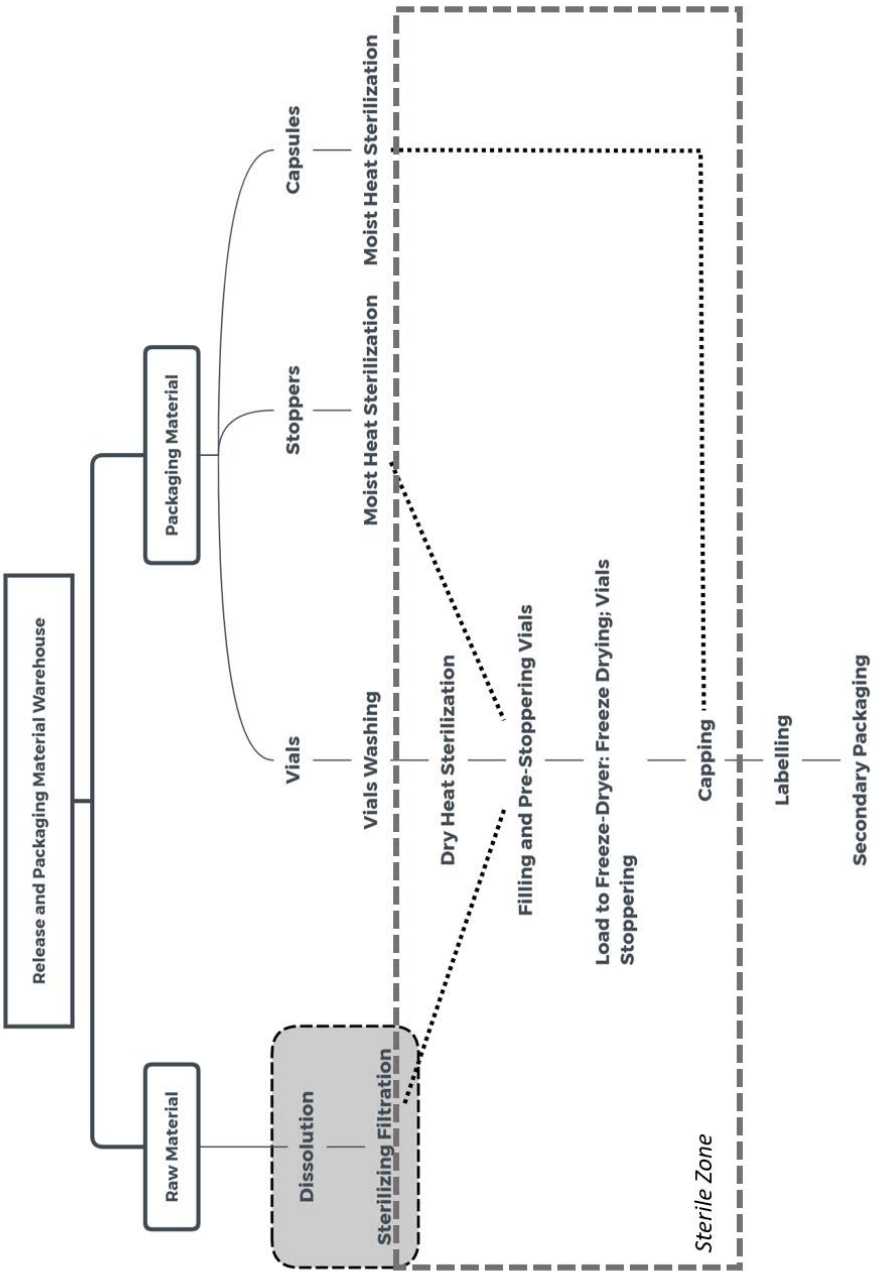

Figure 2 The pharmaceutical manufacturing process of sterile products using sterilization by filtration.

4.1 Types of processes

Manufacturing processes can be divided into the ones that give rise to terminally sterilized products or aseptic processes. Whenever possible, parenteral products are sterilized in its final sealed container and within as short a time as possible after the filling and sealing have been completed. Final sterilization usually involves a thermal process, although there is a trend to apply radiation sterilization to final products, however not all production facilities are prepared for that and some products may lose efficacy. High temperature exposure may be a problem for biologics or some pharmaceutical products, because it may be associated to a loss of stability. For that reason, sterilization by filtration is used when an aseptic processing is required. The successful production of sterile parenteral drugs by aseptic processes need an extreme control over the environment, in order to avoid particle entrance and microbial contamination. Aseptic processing consists of sterilizing different components separately and mixing them at the end, filling in their final container in aseptic conditions[4,5].

Control of aseptic processing has changed over the last 50 years, being one of the most important events the appearance of HEPA filters in the late 1950s. As it already was generally accepted that personnel is the main source of contamination, physical separation between the operator and the critical zone had been used. After that, use of classified areas by air flow and pressure differences was introduced. Finally, the recent use of isolators has returned the idea of physical separation as a contamination prevention measure[4].

4.2 Manufacturing facilities

Facilities involved in the manufacturing of pharmaceuticals must be designed, constructed and operated correctly for the production of a sterile product with the required level of quality for its safety and effectiveness. Materials of construction for facilities need to be smooth, cleanable and must avoid moisture. Air passages and closures must be controlled in order to avoid particle and microbial contamination[5].

Rooms in which sterile manufacturing activities are being carried out are classified according to air cleanliness, which corresponds to its particle content, viable and non-viable. The classification of the clean areas is consistent with the level of nonviable and viable particulate contamination acceptable with the activity conducted in the facility. Because so many sterile products are manufactured at one site for global distribution, air quality standards in aseptic processing areas must meet both United States and European requirements. European requirements use grade classification (from A most critical and clean, to D less critical) while United States relies on ISO classification that uses Classes, named after nonviable particles accepted[5,4]. Table 1 summarizes the different specifications for each classified area, relating European and United States nomenclature.

Table 1. Classification of clean rooms according to particle and microbial content[6,7]

		Particle Count		Microbial Contamination	
Class[a]	Class[b]	AR[c]	IO[d]	Active air sampling[e] (CFU/m^3)	Settle Plates (CFU/4h)[f]
A	ISO5 (100)	3,500	3,500	<1	<1
B	ISO6 (100)	3,500	35,000	10	5
C	ISO7 (10000)	350,000	3,500,000	100	50
D	ISO8 (100000)	3,500,000	N/A	200	100

[a] In accordance with GMP Annex I.
[b] In accordance with US Federal Standard 209EISO 14644-1.
[c] AR: At Rest conditions
[d] IO: In Operation conditions
[e] 10 minutes of air sampling with slit-to-agar samplers
[f] 90-mm settling plate. These are average values and individual plates may have <4 h of exposure.

Flow of equipment, materials and personnel needs to move from lower classified environments to higher classified environments, as represented in Figure 3.

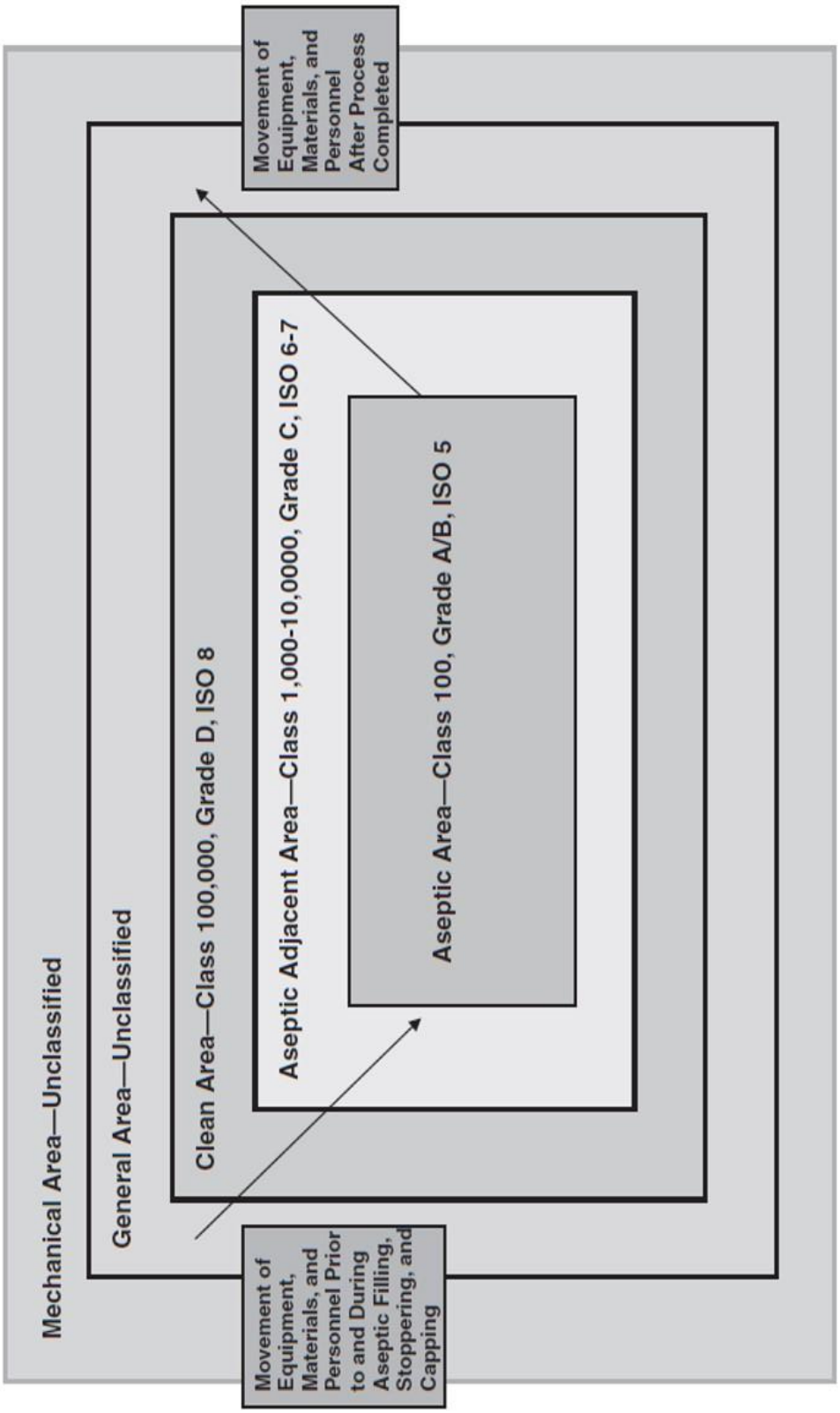

Figure 3. Air flow direction in classifies areas[5]

Activities to be performed in each classified area are determined according to its level of criticality, being the most critical parts of the process the ones in which the product is exposed to the environment and in consequence susceptible to microbial contamination. Activities to be carried out in the different classified areas depend on the type of process, terminally sterilized products or aseptic processes. However, the common factor is that the most critical operations take place in the highest level of classification.

For example, in a general context, grades C and D are preparation zones, in which solutions are prepared before filling and filtering, grade B zones serve as background for grade A areas and grade A are designed for aseptic preparation, if needed, of products and filling.

Figure 4 shows the airflow direction in a sterile manufacturing facility. The activities that take place in each classified clean room are indicated, being preparation the less restrictive activity. Filling is always the most critical activity as the product is introduced in its final package and it is exposed to the environment. Black and red arrows indicate the airflow direction. However, the flow of the process moves in the opposite direction. to the airflow.

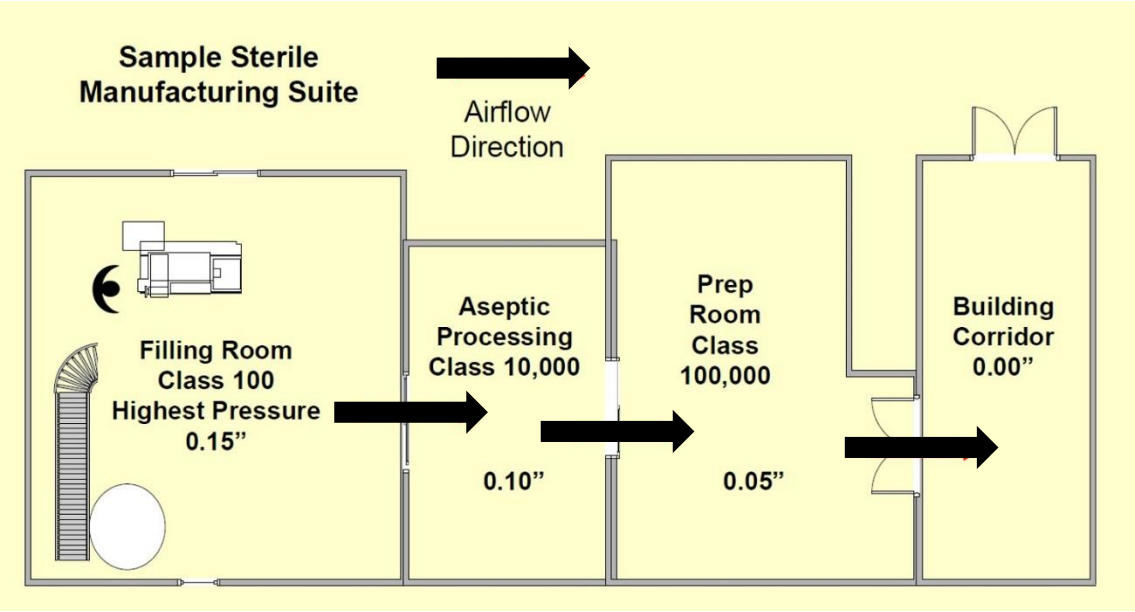

Figure 4. Clean room layout of a sterile pharmaceutical manufacturing facility[8]

5 Regulatory framework

5.1 Good Manufacturing Practices

One of the critical points in pharmaceutical industry is quality control of the manufacturing process and the final product, along with quality assurance. All activities taking place in the pharmaceutical industry are subdued to current Good Manufacturing Practices (cGMP).

Medicines regulation requires scientific knowledge and skills in the medical, pharmaceutical, chemical or biological fields, among others; all applied within a legal framework to serve patients[9]. There are different aspects that define quality in a pharmaceutical product, not only physicochemical properties are essential but microbiological properties may also affect efficacy and safety of products[3].

Good Manufacturing Practice regulations were first proposed by the United States government in 1963, following congressional passage of the Kefauver–Harris amendment in 1962, in turn, following the thalidomide tragedy in the United Kingdom in 1960–1961. These regulations described the basic requirements for the manufacturing, packaging and distribution of finished pharmaceutical products[5].

Compliance to cGMP ensures that pharmaceutical products taken by or administered to humans and animals meet or exceed minimum requirements of safety, identity, strength, purity and quality[5].

Authorities requiring cGMP compliance for pharmaceutical manufacturing industries mainly include the European Union (EMA), the UK Medicines and Health Care Products regulatory agency (MHRA), Pharmaceutical and Medical Devices Agency in Japan (PMDA), Pharmaceutical Inspection and Co-operation Scheme (PIC/S), WHO and the US Food and Drug Administration (FDA)[3].

cGMP defines quality measures for both production and quality control and defines general measures to ensure that processes necessary for production and testing are clearly defined, validated, reviewed, and documented. In addition, the personnel, premises and materials must be suitable for the production of pharmaceuticals and

biologicals including vaccines. GMP has also legal components, covering responsibilities for distribution, contract manufacturing and testing, and responses to product defects and complaints. Specific GMP requirements relevant to classes of products such as sterile pharmaceuticals or biological medicinal products are provided in a series of annexes to the general GMP requirements[3,10].

cGMP are defined by the FDA in the Code of Federal Regulation[11] and in the European Union they are described in Eudralex "EU Guidelines to Good Manufacturing Practice: Medicinal Products for Human and Veterinary Use"[12]. Because GMP were firstly proposed as law in 1963 with relatively minor changes to the current ones, it is important to distinguish "c" for *current* GMP and they are now referred as cGMP.

GMP compliance must not be seen as a regulatory burden. Failure in accomplishing GMP may have a critical massive impact on patients' health and further economic consequences on the manufacturer, as litigation or recall on product may occur[3]. Nevertheless any inspection conducted by Regulatory Authorities refers continuously to the criteria stablished in the cGMP.

Pharmacopoeia is an official publication that lays down quality standards for medicinal products. They can be national like the British Pharmacopoeia or the United States Pharmacopoeia or regional like the European Pharmacopoeia. A Pharmacopoeia contains a number of general texts, as well as monographs for individual drug substances and finished dosage forms. They describe a set of tests that will confirm the identity and purity of the substance or product, the amount of active substance and related substances (impurities) contained in it and other characteristics like dissolution or disintegration properties. A Pharmacopoeia thus enables independent quality control of drug products and drug substances to test if they accomplish the required technical criteria.

Mainly, European companies follow European Pharmacopoeia (EP) guideline documents and monographies. Whereas, companies which wish to enter the American market must also meet requirements of United States Pharmacopoeia (USP) for their products. In the case of microbiology, every Pharmacopoeia has its specific chapters describing requirements for microbiology methods validation as well as specifications for every

assay, which will be described in a following section. Content in different Pharmacopoeia are very similar, although they may have slight differences. When validating a technique, in order to meet requirements for both Pharmacopoeia, validation protocols must be designed regarding the more restrictive one and trying to cover as much analysis as needed by both Pharmacopoeia.

Regarding sterile products, specifically parenteral ones, Parenteral Drug Association (PDA) is a reference organism. This organization has different documents in the form of technical reports that describe and give recommendations on how to perform different activities regarding pharmaceutical microbiology that are aligned with the FDA requirements. For example, Technical Report (TR) 13 on "Fundamentals of Environmental Monitoring Program"[13] or TR 33 on "Validation of alternative methods"[14] are very useful on implementation of microbiology techniques as they help users in the interpretation of Pharmacopoeia. Nevertheless, it is important to bear in mind that PDA documents are not regulation bounding, they are only informational.

6 Quality in the Pharmaceutical Industry

Ensuring the quality of the final manufacturing products is one of the main tasks of the Pharmaceutical Industry and all necessary efforts must be taken in order to achieve it. For that, a quality system that integrates different activities is designed in every company.

As shown in Figure 5, analysis performed during In-process control (IPC) and quality control (QC) laboratories of the manufactured products must follow GMP guidelines. Quality assurance (QA) in the company must ensure the correct application of GMP in

the different areas. All of this is integrated in the quality system that is involved in the manufacture of a product.

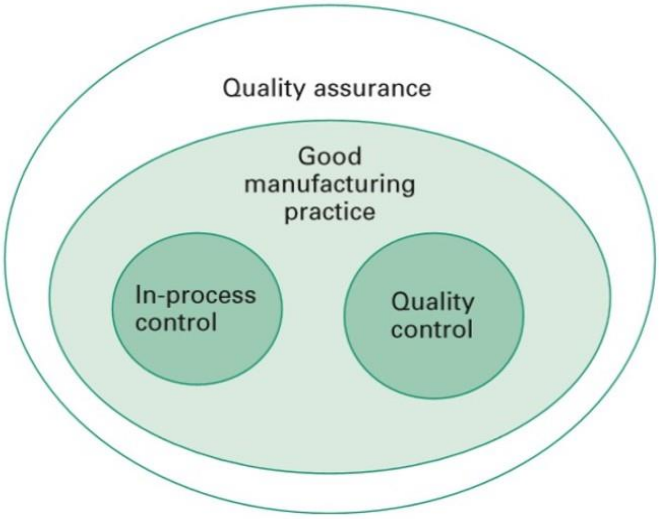

Figure 5. Interrelationship between quality assurance and quality control[3]

6.1 Quality Control

Quality control (from now on QC) is responsible for reviewing the batch history and performing in-process control testing, final product testing or environmental monitoring.

Specifications for every assayed parameters are set during development and validation of manufacturing process of every product, according to Pharmacopoeia or other guidance such as International Standardization Organization (ISO) or for example PDA.

QC department carries out the evaluation of every batch release parameters in order to ensure that the product is safe, effective and attributes are reproducible from batch to batch. In addition, stability studies for marketed products are performed by QC to ensure that product properties are maintained through time.

6.2 Quality assurance

Quality assurance (from now on QA) covers all aspects of quality that include QC, manufacturing, distribution and inspections. Role of QA has changed in part as a consequence of International Conference for Harmonization (ICH) Q10 publication,

where the need of establishing a Quality System appeared[15]. A quality system must include the following characteristics:

a) Establishing, implementing and maintaining a process that ensures that products meet quality required by patients, health professionals and health authorities.
b) Establishing and maintaining a state of control using effective process performance and product quality monitoring systems.
c) Promotion of continuous improvement of product quality and manufacturing processes.

The major functions of a quality system are process performance and product quality monitoring system, reviewing, applying corrective and preventive actions, in order to be in continuous improvement. In addition, quality must be based in scientific principles and oriented to risk management.

6.3 Quality by Design

Pharmaceutical industry quality system has always been based on the "quality by inspection" system, which is not a risk management position. However, there is now a change of vision from "quality by testing" (QbT) to "quality by design" (QbD). This change of perspective is being adopted by regulatory organisms such as FDA and many companies are moving through this new way of understanding and managing quality.

Pharmaceutical QbD is a systematic, scientific, risk-based, holistic and proactive approach to pharmaceutical development. It consists of identifying critical characteristics that affect quality of products, translating them intro attributes that the product should possess and establishing how the critical process parameters can be varied in order for the product to achieve the desired characteristics[16].

FDA has developed a question-based review for its chemistry, manufacturing and controls (CMC) evaluation of abbreviated new drug application (ANDA). This initiative consists of a quality assessment system that is based on critical pharmaceutical quality attributes which incorporates some elements of QbD.

The change affects from research and development, going through manufacturing to regulatory concerns. To configure the production capabilities required for this new era of manufacturing, decisions need to be made on products, technologies and sites.

There are several steps to follow when implementing QbD in a pharmaceutical manufacturing process, being one of them identification of critical points along the process (products critical quality attributes). Pharmaceutical microbiology tests are critical in the release of final products, as most drugs are susceptible to the deleterious effects of microorganisms, especially in the case of sterile products.

The most critical tests regarding microbiology are sterility testing and testing for pyrogenicity. As limitations of the sterility tests have been identified and discussed[17], important focus is given to sterilization process and validations of the same. In any case, efforts must be addressed to minimize the microbial content of the product along the manufacturing process.

6.3.1 Parametric release

Parametric release techniques, which are more commonly named as Process analytical technology (PAT), is an initiative to support QbD. FDA published its Guidance for Industry describing PAT initiative[18] in order to encourage industries to take a risk-management posture and give a regulatory framework for the implementation of these innovative technologies. PAT provides a better understanding of the process which will add more flexibility in order to improve it with time.

In the case of microbiological attributes, PAT will permit to release terminally sterilized products without the need of a sterility test, however, terminal sterilization process must be validated and demonstrate that critical parameters of sterilization are met[19].

7 Pharmaceutical Microbiology

7.1 Microorganisms of importance in the Pharmaceutical Industry

Importance of microorganisms in the pharmaceutical industry is mainly because of infections they can cause in patients and possible spoilage of the products.

Bacteria have the ability to grow on different substrates which make them potential spoilage candidates of raw materials and products. Even some species are able to survive drying processes and are capable to resist in dust, becoming important contaminants of manufactured medicines. The ability of microorganisms to survive not only dry environments but also adverse conditions, such as heat or radiation, is well exemplified by bacterial spores and some fungal spores, although they aren't as resistant as those from bacteria. Such is the survival resistance of bacterial endospores that they are used as sterilization process indicators[3].

As part of the rise in quality standards in the pharmaceutical industry, demonstrating evidence of microbial safety is crucial in product batch release. Microbiology plays an essential role though in aseptic manufacturing processes and sterile products.

Microorganisms are not only a concern in pharmaceutical manufacturing because of pathogens but because sometimes microbial contamination can cause spoilage of pharmaceuticals by chemically degrading the active pharmaceutical ingredients (API)[3], being this last factor less predictable. In that matter, pharmaceutical microbiology must encompass the subjects of sterilization and preservation against microbial spoilage, necessarily knowing which contamination sources are and what factors predispose or prevent spoilage.

Figure 6 shows the most common sources of microbial contamination of pharmaceutical products. Microorganisms can be introduced in the pharmaceutical process through different points, from starting materials to personnel working in the facility. In order to avoid contamination, disinfection of materials and equipment is performed, as well as air filtration and sterilization of the manufactured products. All processes must be

validated in order to ensure their effectiveness, however, some microorganisms are capable of surviving stressing conditions, as mentioned before.

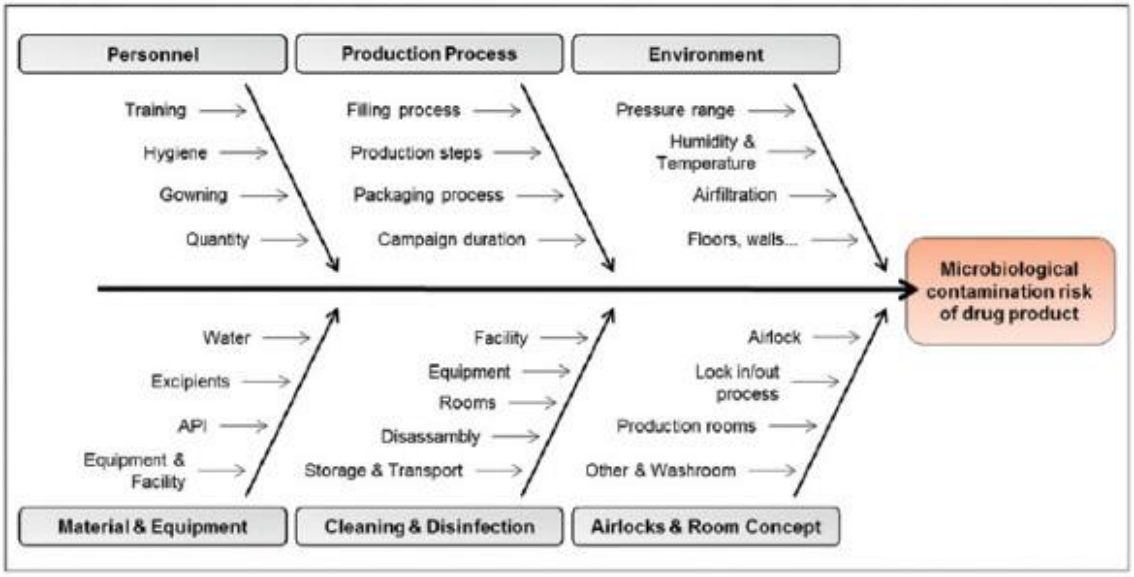

Figure 6. Ishikawa diagram of the most probable sources of microbial contamination of pharmaceutical products[20]

Microbial testing is present during the entire production process as well as in final product testing and a wide variety of analysis need to be performed.

Figure 7 shows the layout of pharmaceutical quality control of the manufacturing process and products. At the center of the image microbiological tests performed are shown. Environmental monitoring of the process is made from mixing of raw materials and water to finished product filling. In between manufacturing activities, samples are taken and microbiological laboratory controls are also performed.

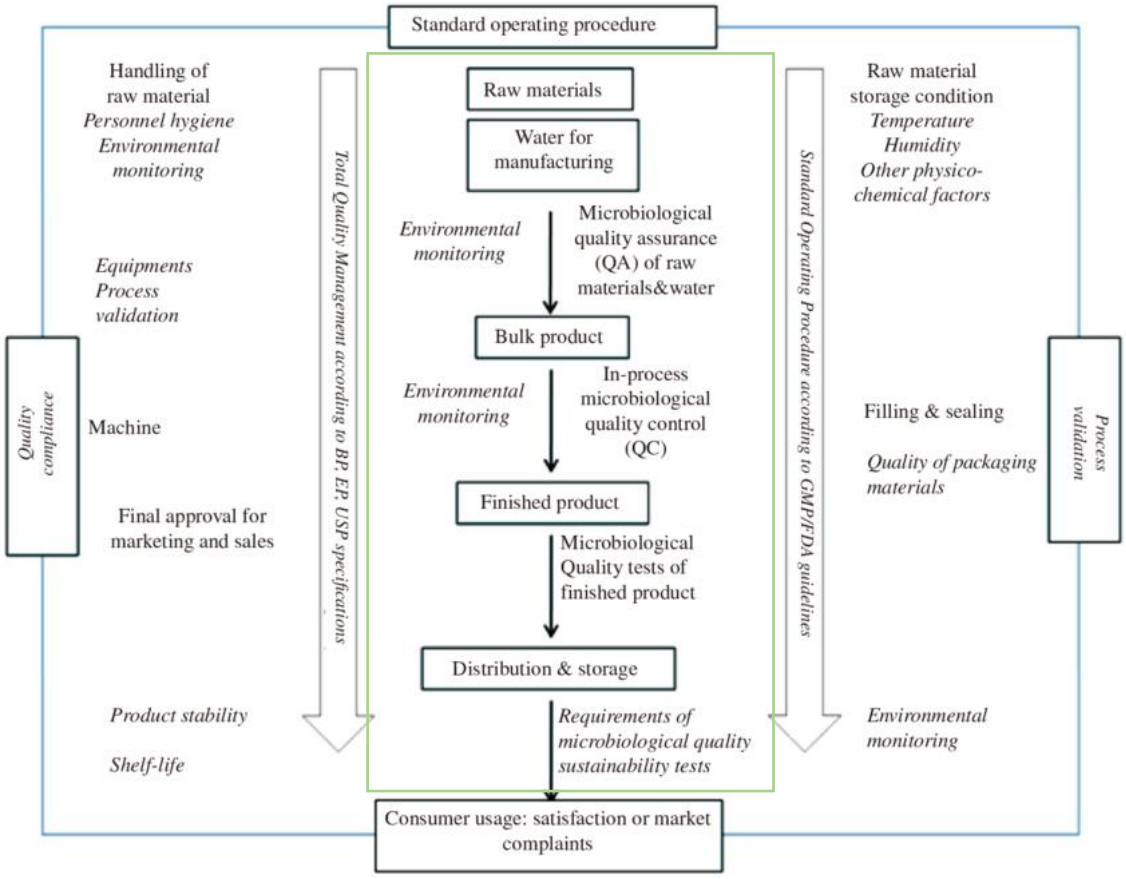

Figure 7. Microbiological requirements and tests of the pharmaceutical process and products[18]

Regarding microbiological tests, they can be classified in two groups: tests for controlling the manufacturing process and product testing. In this section, microbial tests to be performed in pharmaceutical processes and products are described.

7.2 Microbial control of the process

GMP guidelines state that manufacturing areas must be clean environments in order to ensure safety of the final product and because of that, environmental monitoring is necessary along manufacturing process and manufacturing areas. Environmental microbial monitoring provides data and confirms the effectiveness of microbial controls present in the manufacturing and testing areas.

Environmental monitoring is critical in areas where sterile products are being manufactured and a monitoring program must be established, defining critical sampling points, on the basis of a risk analysis. A large proportion of sterile products are

manufactured under the same aseptic environment. Maintaining an aseptic environment consists mainly on the prevention of microorganisms from entering open containers during filling. For that reason, product bioburden as well as microbial bioburden of the manufacturing environment are important factors relating to the sterility assurance of the manufactured products[22,23]. Figure 8 summarizes when sampling for microbial monitoring of the process takes place.

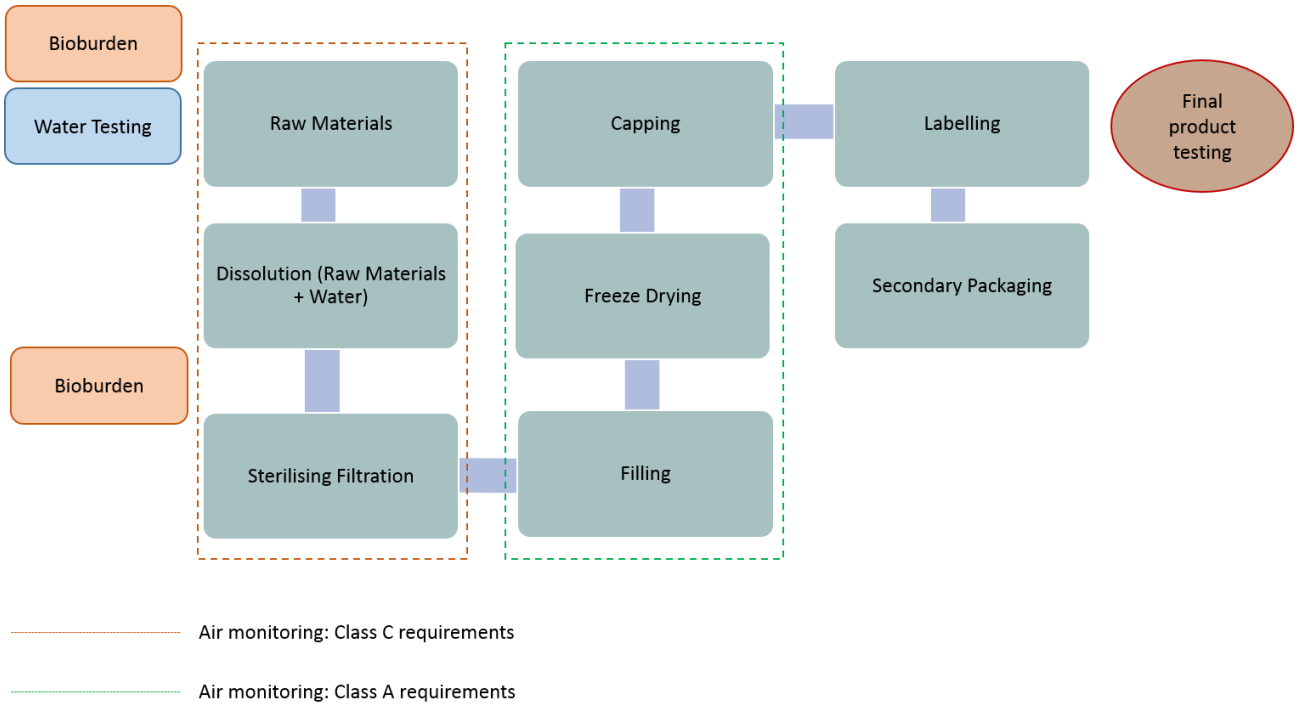

Figure 8. Schematic summary of the microbiological monitoring points in the pharmaceutical manufacturing process

7.2.1 Microbial monitoring of clean rooms: air and surfaces

A clean room is commonly a term used to designate a controlled space free of dust and other contaminants, where number of particles present in the air can be monitored and under contention status. As previously explained in previous section of Manufacturing facilities, clean rooms are classified according to their particle content, being the cleanest the ones with less particles, where most critical operations take place.

One major part of environmental monitoring consists of particle count of cleanrooms, as particles can be potentially a microbial contamination. Nevertheless, particle

monitoring doesn't give the information of the total microbial count of a cleanroom, although microbial bioburden of the room must be assessed and ensured minimum.

Traditional methods for assessing microbial contamination are recovery of microorganisms in culture media and incubation for growth detection [22-26]. Air is sampled by dynamic air samplers or static plates used during operations, in order to recover any microbial contamination. Surfaces are also sampled as well as personnel garments, such as gloves, as they can be in direct contact with the product. Plates containing environmental samples are incubated during 3 to 5 days in order to assess any microbial growth. Maximum limits of microbial counts recommended by Annex I of GMP guidelines[10] are shown in Table 1, these limits are the ones necessary to take into account when qualifying a cleanroom.

Apart from traditional methods, nowadays there are alternative rapid methods which are faster in monitoring microbial contamination thanks to an up-to-date technology, which is, in addition, more sensitive than traditional methods. Rapid microbiological methods will be explained later on in this text.

Special attention is paid in personnel training. Personnel working in aseptic facilities must be properly trained and be aware of the importance of hygiene and cleanliness, as personnel are one of the major sources of microbial contamination. So is, that strict control of garments, such as dressing and gloves, are sampled for microbial detection too.

However, a monitoring system doesn't quantitate and identify all microorganisms present in a cleanroom, although they must provide enough information about the state of the same in order to ensure that it is under control parameters. The aim of microbial monitoring is to obtain representative estimates of the bioburden of the environment. In base to this information, analysis must be performed in order to detect any possible trend. Data from long periods of time must be collected and trends can shed light into the origin of a problem to meet control parameters. If trends are detected, an investigation must be initiated in order to prevent a major problem and to get back to control limits[26,13].

An environmental control program should be capable of detecting any adverse drift in microbiological conditions in a timely manner that allows for meaningful and effective correction action. Because of that, one of the main interests in this work has been implementation of a rapid method for microbial environmental monitoring, which will be discussed later in this text.

7.2.2 Water for pharmaceutical purposes analysis

Control of water for pharmaceutical purposes is essential as it is one of the raw materials used in the manufacturing of pharmaceuticals. In addition, it is part of the circuitry of pipes in the manufacturing plant and is in contact with many surfaces of different locations, so it may be a very typical contamination source. So, apart from a series of physicochemical properties that water for pharmaceutical purposes must comply, it must meet a series of microbiological requirements.

Water used in the pharmaceutical industry can be classified into different types and every type can be obtained by different methods and from different sources[27]. Table 2 summarizes the types of water produced and used in a pharmaceutical manufacturing facility.

Table 2. Types of water for sterile manufacturing[3]

Type	*Properties*	*Use*
Mains (potable)	Non sterile, contains ions, chlorine	Initial washing if rinsed with purified
Purified water	Potable water purified by distillation, ion exchange, reverse osmosis	Non sterile Washing containers
Water for injections in bulk	Distilled water, free from pyrogens	Final rinse Solutions to be sterilized
Sterile water for injections	Autoclave in suitable containers	Sterile solutions

Especial attention will be paid to water for injection as it is used as an excipient in the manufacture of sterile products. In this type of water, level of endotoxins must be highly reduced in comparison to other products, as they enter patient bloodstream directly. Microbial burden present in water for injection is the minimum permitted (10 CFU/100

ml). Nevertheless, water for other uses must be also controlled as they are one of the main sources of contamination and they can be in contact with aseptic material.

Water testing consists of the same analysis regardless the type of water that is being analyzed, but the specification for the result obtained is different in every case. In the case of water for injection, as stated before, microbial count must be not higher than 10 CFU/ 100 ml while specification for purified water is 100 CFU/ 100 ml. The amount of sample to be tested must be indicated in the water monitoring program. Regulation states that at least 100 ml of the sample must be filtered, however, a higher volume may be necessary in the case of water for injection as the low bioburden present in water might difficult detection of contamination.

Enumeration of microorganisms is commonly performed according to the traditional method that will be described later in this text, although there are alternative rapid techniques available that can be used after being properly validated against the compendia method.

7.2.3 Bioburden

Bioburden is commonly used in the pharmaceutical industry to refer the microbial load. Sterilization taking place during or at the end of the manufacturing process is intended to eliminate all bioburden that might be present in products. For that, it is preferable that starting material used in the process has minimum bioburden as it is easier to sterilize.

Bioburden is necessarily undertaken by the supplier of the starting material, although it may be retested upon receiving at the plant before manufacturing. Different Pharmacopoeia describe bioburden requirements that must be accomplished by starting material manufacturers. In addition, prior to sterilization, bioburden is performed of the manufactured product at this point. It is important to achieve a minimum bioburden during the manufacturing process in order to ensure complete sterilization. Low bioburden at this point is achieved by not only using starting material with low bioburden but it also depends on the strict control of the manufacturing process[28,29].

There are different strategies to maintain a low bioburden and avoid microbial entrance and grow on manufactured products. Water, as the main source of microbial contamination, is usually kept at a temperature above 80°C. In addition, factors such as pH are important in order to avoid microbial growth [3].

7.3 Microbial control of products

Manufactured final products are tested before release in order to ensure that specifications set during process validation are met. In the case of microbial testing, different analysis are carried out depending on the type of product. Tests performed routinely in the microbiology laboratory of a pharmaceutical company are described below.

7.3.1 Antimicrobial Effectiveness Testing or Microbial Limits Test

This test is commonly known as microbial challenge test. Antimicrobial preservatives are substances that can be added to sterile and non-sterile products in order to prevent the growth of microorganisms that can be introduced inadvertently during or after the manufacturing process. However, the addition of preservatives cannot be used as substitutes of good manufacturing practices. Antimicrobial effectiveness test is designed to demonstrate the antimicrobial properties of these preservatives. The test is performed by inoculating different microorganisms with the product and culture media and letting them incubate for 3 to 7 days, checking that the population initially inoculated hasn't increased[30].

7.3.2 Microbial examination of Non-Sterile products

Non-sterile products, starting material as well as final products, need to meet specified microbial quality criteria, as stated in the different Pharmacopoeia. Basically, examination of non-sterile products consists of microbial enumeration. This test is traditionally performed by inoculating the product into culture media and incubating it for 3 to 5 days in order to check if microbial limits are achieved. Enumeration of microorganisms is in non-sterile products known as bioburden test. Only aerobic

microorganisms, including bacteria, yeast and molds can be detected in this test. In addition, tests for specified microorganisms are performed as the absence of them is required, for example, *Pseudomonas aeruginosa*[29].

7.3.3 Sterility testing

Sterility test, as described in the Pharmacopoeia, is not designed by itself to demonstrate that a batch of product is sterilized or has been successfully sterilized. This is accomplished primarily by validation of the sterilization process. So, satisfactory sterility tests results indicate that no microorganism was found in the sample examined under the conditions of the test. Nevertheless, it is a required test for batch release in sterile products and it is the longest test in time. Samples are filtered and inoculated in the appropriate media culture, afterwards they are incubated between 7 and 14 days in order to determine absence of microbial growth[31].

7.3.4 Bacterial endotoxin testing

Endotoxins are commonly known as pyrogens, which are molecules that provoke a rise of body temperature, normally produced by gram-negative bacteria. The principle of the endotoxin test is based on amoebocytes present in the horseshoe crab bloodstream, acting as a defense mechanism producing a clot when in contact with a pathogen. The test described in the Pharmacopoeia consists of the reaction between the endotoxins and the amoebocytes, adapted as a synthetic reagent. The resulting reaction can be measured as a gel-clot formation or by a photometric assay. Limits for endotoxin tests are defined on the base of dose, which depends on the threshold human pyrogenic dose of endotoxin per kg body weight and the recommended human dose of product per kg of body weight[32].

7.4 Identification of microorganisms

Isolates found during microbial monitoring of both processes and products need identification. An identification plan is necessary in order to establish which isolates need identification and which level is required.

Every microbial monitoring plan should include an appropriate level of identification of the microorganisms detected. Knowing the microbial diversity of the plant aids for determining effectiveness of cleaning and sanitation. In addition, a proper identification plan helps to gather information during investigations when action levels are achieved to assess the source of contamination. With the new revision of annex I of GMP guidance[10], every microorganism found in areas A and B must be identified to species level[6].

Identification of microorganisms in the pharmaceutical industry and the different methods available will be explained later on.

8 Traditional microbial testing in the pharmaceutical industry

Traditionally, microbial testing performed in the pharmaceutical industry, for the vast amount of microbial tests described in section 7, is based on culture techniques.

As described before, there are different types of microbial tests: they can be quantitative or qualitative. Qualitative tests are based on presence or absence of growth (sterility test), while quantitative tests are based on enumeration of microorganisms (for example, bioburden test).

8.1 Culturing techniques

Most bacteria and some yeasts divide by a process known as binary fission whereby the cell enlarges or elongate, then forms a cross-wall (septum) that separates the cell into two equal compartments that share the same copy of genetic material. Septum formation is followed by constriction in which the connection between the two compartments is progressively reduced, until it is finally broken originating two individual cells. In bacteria, this pattern of division lasts about 25-30 minutes under optimal conditions of laboratory culture[3].

Starting from a single cell, many bacteria can achieve concentrations of about 10^9 cells/ml or more following overnight cultivation in liquid media. At concentrations below about 10^7 cells/ml culture media are clear, but the liquid becomes progressively more

turbid as the concentration increases above this value and indirect measurement of microbial growth can be performed[3].

When growing microorganisms in solid media individual bacterial cells can give rise to colonies following overnight incubation under optimal conditions. Colonies are usually named as Colony Forming Units (CFUs), which are typically the units in which quantification results are expressed. It is assumed that 1 CFU is originated by a single cell and colony size is limited by nutrient availability and/or waste product accumulation in just the same way as concentration in liquid media[3].

Anaerobic organisms may be grown on Petri dishes provided that they are incubated in an anaerobic jar. Such jars are usually made of rigid plastic with airtight lids, and Petri dishes are placed in them together with a low-temperature catalyst.

Generic media used in the pharmaceutical industry for microbial testing is Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB) for aerobic bacteria, Thioglycolate for anaerobic microorganisms and Saboureaud Dextrose for fungi, both yeast and molds. There are specific culture media depending on the necessities, for example, in order to recover stressed microorganisms from water of pharmaceutical use a less nutritive media such as R2A is used, in order to reduce stress factors for growth.

8.2 Enumeration of microorganisms

In pharmaceutical context, as described before, it is necessary in several situations to measure the number of microbial cells in a culture[3]. In some cases, the total number of cells, both dead and living cells, is necessary as dead cells can act as pyrogens, although in most cases only the number of viable cells (understood as living cells) is required. The terminology in microbial counting can be confusing: a *total count* is a process of enumerating both living and dead cells, whereas a *viable count*, more common in pharmaceutical tests, only takes into account living cells. However, the term total viable count (TVC) is used in most Pharmacopoeia and regulatory agencies to mean a viable count that records all microbial species or types of microorganisms that might be present in a sample, for example bacteria plus fungi[3].

Microbial enumeration is most commonly performed by the ability of microorganisms to form visible colonies in a plate after incubation, so colonies can be counted manually and results are given in CFU. When the expected number of colonies is low, membrane filtration is used as a previous step to concentrate microbial cells and the membrane itself is incubated on the surface of the agar plate. Another method is the Most Probable Number, which is also used when expected counting is relatively low. The procedure involves inoculating multiple tubes of culture medium (usually three or five) with three different volumes of sample. If the concentration of the sample is between 1 to 100 CFU/ml, there will be a number of tubes with inoculum in which no microorganisms will be present. The number of tubes with no growth is compared with standard tables showing the most probable number per milliliter. However, this technique is not as used as plate counting in the pharmaceutical industry, although is well accepted in Pharmacopoeia[3].

8.3 Constraints of traditional methods

Traditional methods for viable counting suffer from the same limitations:

- Relatively labor intensive
- Difficult to automate
- Slow, because they require an incubation period for colonies to develop or liquid cultures to become turbid
- May require relatively large volumes of culture media, many Petri dishes and a lot of incubation space

The precision, accuracy and standard error of classical microbiological methods is affected by the distribution of microorganisms in the test samples, cellular arrangement, sample dilution and the plate count method itself. Such that the antimicrobial or preservative effectiveness test error is ±0.5 $\log_{10}$[14] and standard plate count errors range from 18% to 100% (error as the percentage of variability of the mean of counts) when the mean number of colony forming units per plate is 30 to 1, respectively[33].

Additionally, microorganisms that are stressed due to nutrient deprivation following exposure to lethal concentration of antimicrobial agents, such as preservatives,

disinfectants, heat, or decontaminating gases or as a result of certain pharmaceutical manufacturing processes, may be stressed or physiologically injured. These microorganisms are not dead because they can still perform some metabolic activities, but they cannot grow on culture media because the media used may not be optimal for resuscitation of these microorganisms and recovery[34,35]. The vast majority of microorganisms of importance in pharmacy and medicine can be cultivated in the laboratory and most of cultivation techniques are simple and require few technological equipment[3]. However, viable but non culturable microorganisms are a known fact and has been taking importance in the pharmaceutical industry as every time more information is available about this state and the consequences it can have in human health.

The fact that traditional growth-based methods are very long (ranging from 5 to 14 days depending on the test) is a determining factor on batch release time. In addition, when contamination is found the batch is in a late stage of the process, making difficult to find the root cause of a contamination. It sometimes ends up in batch rejection which causes an economic loss for the company.

For these reasons, interest in developing and implementing so-called “rapid” methods in microbiology has increased in the last decade[3].

9 Rapid microbiological methods (RMM) in the pharmaceutical companies

Rapid microbiological methods (RMM) permit a faster detection or identification of microorganisms in comparison to traditional methods, mostly based on culture and long incubation periods, as explained in the previous section. In the research context, advanced technology for microbiological detection is being used for a long time, however industries have been more reluctant to change due to the regulatory framework they are surrounded by.

Traditional microbiology methods used in the pharmaceutical industry are mainly based on sample filtration or direct inoculation (for non-filterable samples) in liquid or solid media and incubation periods between 5 to 14 days, depending on the type of analysis,

to finally detect contamination. When using liquid media, for example in sterility test which is a qualitative method, the presence or absence of turbidity is assessed. While in bioburden or other quantitative techniques performed in solid media, the number of colonies is determined.

A rapid method is defined by having a set of characteristics: they are rapid, possess high sensitivity and precision and they are generally automatized. The principles of rapid methods have been long known, but in the last years equipment has been developed in order for industries to apply them to their routine testing[36].

Rapid methods have acquired high importance in food, cosmetic and pharmaceutical industries, although in the last one implementation is being significantly slower. Medical microbiology diagnostic laboratories have been using rapid methods long before the above mentioned industries, as fast time-to-result may be critical for patient health in some cases. However, obtaining faster results also benefits manufacturing companies that need microbiological testing as they can release products faster and time saving translates in economic saving. In addition, using more sensitive and specific technologies for microbial testing increases product quality and safety.

9.1 Concern of pharmaceutical companies in the use of RMM

There are several reasons why pharmaceutical industries are reluctant to change traditional methods for rapid methods:

- Due to the amount of regulation requirements that industries need to meet, there is a misconception that regulatory agencies will not accept changes. However, FDA, EMA and other organisms have encouraged the application of RMM to microbial testing and different chapters in their respective Pharmacopoeia have been published regarding validation of alternative methods (see section 3).
- Implementation of rapid methods may need a high initial investment on equipment and technology and return of investment is not evident if inventory cost reduction is not taken into account in the economic evaluation. In addition, cost/sample of rapid methods is sometimes higher than that of traditional

methods. However, for example application of rapid methods to in-process testing, faster time-to-result may lead to prevent contamination of final products and batch rejection, which finally translates in economic saving.

- Specifications in Pharmacopoeia are given in CFU and sometimes RMM unit results are given in a different unit. For example, in luminescence based methods, results are given in relative light units. However, evaluation and establishment of new alert and action limits for alternative technologies is part of validation of new methods and it is described in different Pharmacopoeia[37].
- Previous concern is related with the fear that implementation of rapid methods, which are more sensitive, may give rise to an increase in contamination detected up until the moment of implementation. As traditional methods are based on growth results and 1 CFU is not equivalent to 1 cell, it may lead to an exceeding of actual limits when implementing a rapid method. However, USP is aware of CFU limitations and as implementation of alternative methods is encouraged in chapter <1223> of USP[38], proper validation of the alternative technique may justify the change.

9.2 Benefits from implementing RMM

On the one hand, RMM can reduce product loss, release time and testing and labor costs:

- Reduction on product loss is a consequence of implementation of rapid methods as they permit a faster decision-making in front of contamination, as long incubation periods of traditional methods are eliminated. Thus, they can help preventing a contamination in the final product as manufacturing can be stopped when contamination is detected in the process[39].
- Reduction of product release time is an obvious consequence as rapid methods permit a faster time-to-result than traditional methods.
- Reduction of testing cost and labor cost is more difficult to assess as sometimes implementation of rapid methods mean high investment on reagents and equipment. However, as processes are easier to automate, personnel dedication

time is reduced and sample throughput can be increased in order to optimize costs.

On the other hand, implementation of RMM increase product safety, due to several reasons:

- High sensitivity of the techniques in comparison to traditional methods may give a wider knowledge of the microbial status of products and environment that may have been underestimated with traditional methods. For example by the detection of viable but non-culturable microorganisms.
- Fast time-to-result can lead to an improved corrective and preventive action plan in front of contamination, which translates in higher quality and safety of products.

9.3 Types of RMM

Rapid microbiological methods can be classified into two groups: detection methods and identification methods. Figure 9 shows a schematic representation of the most used RMM in the pharmaceutical industry classified in two general groups: detection methods or identification methods, the first group subclassified in growth and non-growth-based methods.

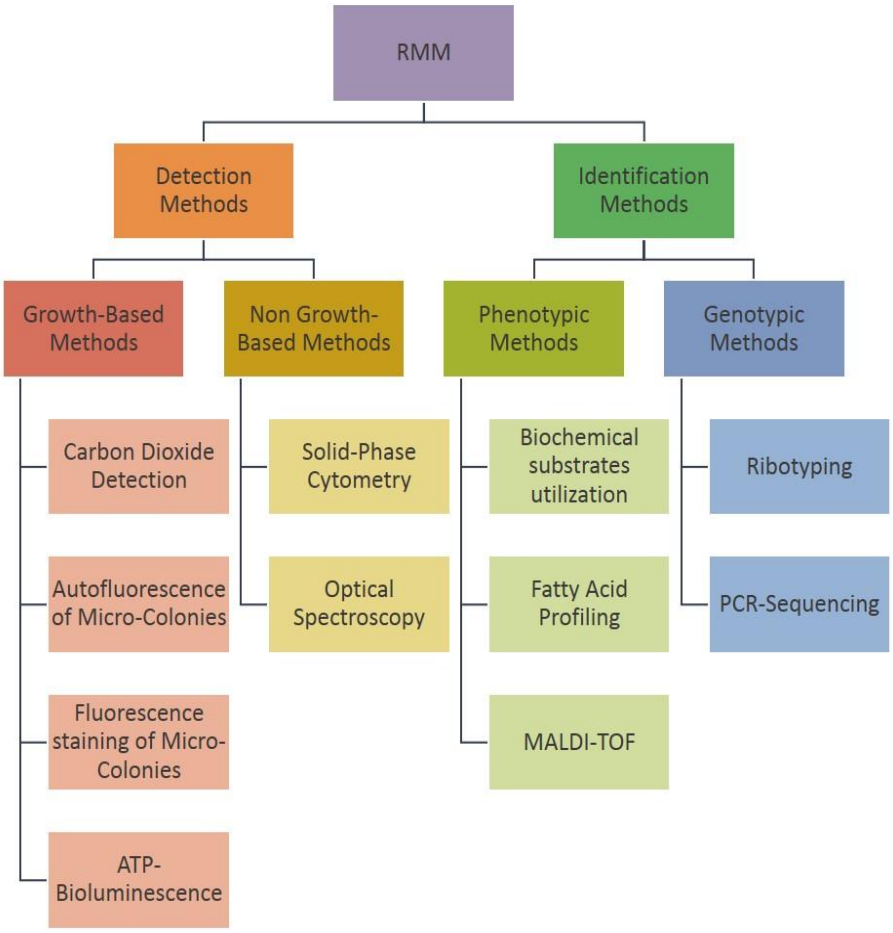

Figure 9. Classification of the most used RMM in the pharmaceutical industry

In this thesis, solid-phase cytometry and optical spectroscopy have been used for detection systems. In addition, MALDI-TOF and PCR-sequencing have been used as identification methods.

9.3.1 Microbial Detection Methods

9.3.1.1 Growth-based methods

Alternative and RMM that employ the use of growth-based platforms have been shown to reduce the time at which actively growing microorganisms can be detected, although the actual time-to-result may be impacted by the physiological state of microorganisms and/or the lag period in which microorganisms adapt to growth conditions. Many growth-based systems continue to use conventional liquid or agar media. As a result, the same types of applications that traditional methods are used for can also be applied

to growth-based alternative and rapid methods. Examples include bioburden testing, Microbial Limits or environmental monitoring[14].

1) Detection of Carbon Dioxide (CO_2)

Microorganisms, when grown in liquid culture, produce carbon dioxide (CO_2) among other metabolites. In a closed container, the amount of CO_2 produced may be monitored and used as a measure of organisms' viability. Test samples are added to media bottles that contain a liquid emulsion or silicone sensor. During microbial growth, CO_2 in the medium diffuses into the sensor. Hydrogen ions will then interact with the sensor resulting in a decrease in pH, and the sensor will turn color. The rate at which CO_2 is detected depends on the initial concentration of microorganisms; for example, a higher initial concentration will provide a faster detection response[14].

An example of a rapid method based of Carbon Dioxide detection in culture media is BacT/Alert system, which has been evaluated for its application in sterility test[40].

2) Digital Imaging and Autofluorescence of Micro-Colonies

During microbial growth, cells will fluoresce in the yellow-green spectral region when illuminated with blue light. Cellular auto-fluorescence in this spectral region is a property of all microbial cells due to the presence of ubiquitous fluorescent biomolecules including flavins, riboflavin'sFlavin, Riboflavin and flavoproteins. Test samples are filtered and the membrane is placed onto an agar surface and incubated. During incubation, a Light Emitting Diode (LED) excites micro-colonies to autofluoresce, which are quantitated by a Charged Couple Device (CCD) imaging system in approximately one-half the time an operator would normally be able to observe colonies on the agar surface. Incubation of the agar can continue to allow for the recovery of larger colonies for subsequent analysis, such as microbial identification. This technique can be applied to filterable samples, such as water for pharmaceutical use or soluble products, to determine its biological burden.

One example of a system using this technology is Growth Direct from Rapid Micro Biosystems, which has been applied to bioburden testing [41]. Figure 10 shows a schematic representation of the mode of operation of the Growth Direct Technology.

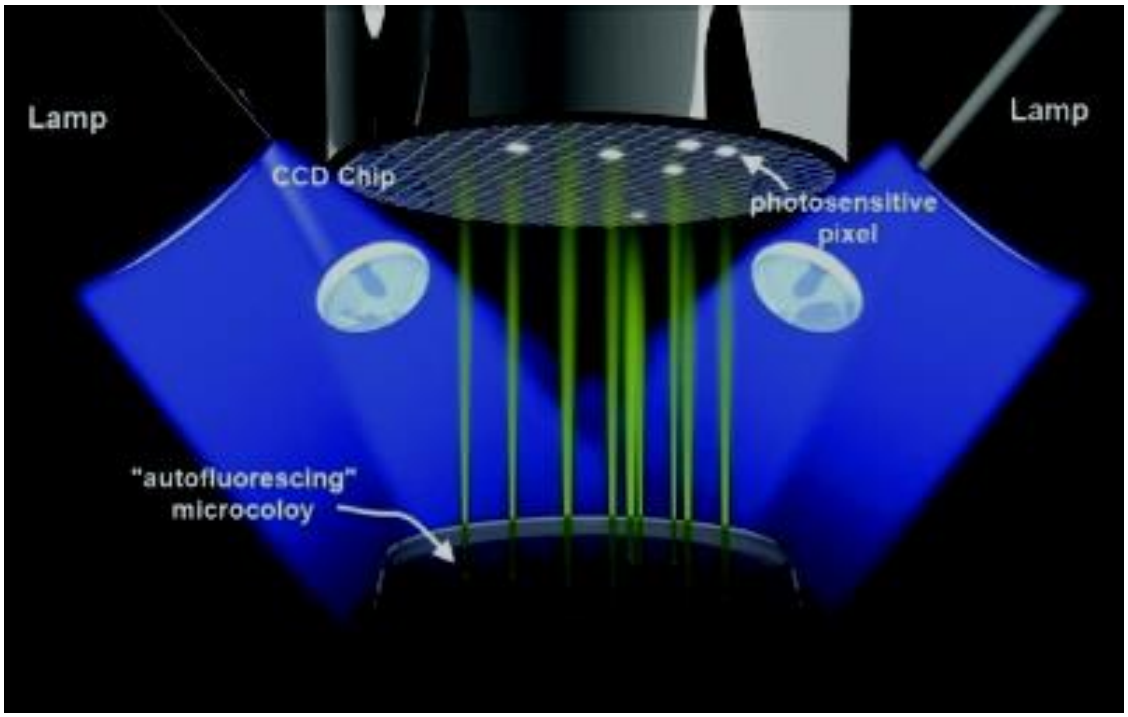

Figure 10. Growth Direct Technology based on auto-fluorescence of micro-colonies[42]

3) Fluorescent staining and laser excitation of microcolonies

Viability staining and laser excitation can also be used to detect and quantify micro-colonies. As well as in the previous methodology, samples are filtered and filters are located on the agar plate surface and incubated. Following an appropriate incubation time, the membrane is stained with a nonfluorescent substrate. Microorganisms on the filter will take up the substrate, which is then enzymatically cleaved, liberating free fluorochrome in the microorganism cytoplasm. As the fluorochrome accumulates inside the cells, the signal is amplified. The membrane is subsequently placed into a reader and exposed to the excitation wavelength of the fluorochrome. Fluorescent micro-colonies are then enumerated. Incubation of the agar can continue to allow for the recovery of larger colonies for subsequent analysis, as it is a nondestructive technique.

4) Cellular component-based technologies: ATP bioluminescence

Cellular component-based technologies rely on the detection and analysis of specific portions of the microbial cell, including adenosine triphosphate (ATP), endotoxin, proteins and surface macromolecules. The applications of these methods are wide and include sterility testing, bioburden and microbial limits testing, environmental and process water monitoring, microbial identification and endotoxin analysis.

ATP bioluminescence consists of the generation of light by a biological process. In the presence of the enzyme luciferase and the substrate luciferin, ATP is enzymatically broken down to produce photons of light. An instrument equipped with a photomultiplier tube can detect these photons. Because ATP is a key intracellular energy source in all cells, measuring ATP can be a marker for viable microorganisms. Depending on the technology, some systems will detect the general presence of microorganisms by measuring the total relative light units from a test sample (Celsis technology), while other systems can detect ATP bioluminescence from individual micro-colonies (Merck technology), thereby providing a quantitative assessment of the number of microorganisms from the original sample under evaluation. Figure 11 shows a schematic representation of the biological process described above.

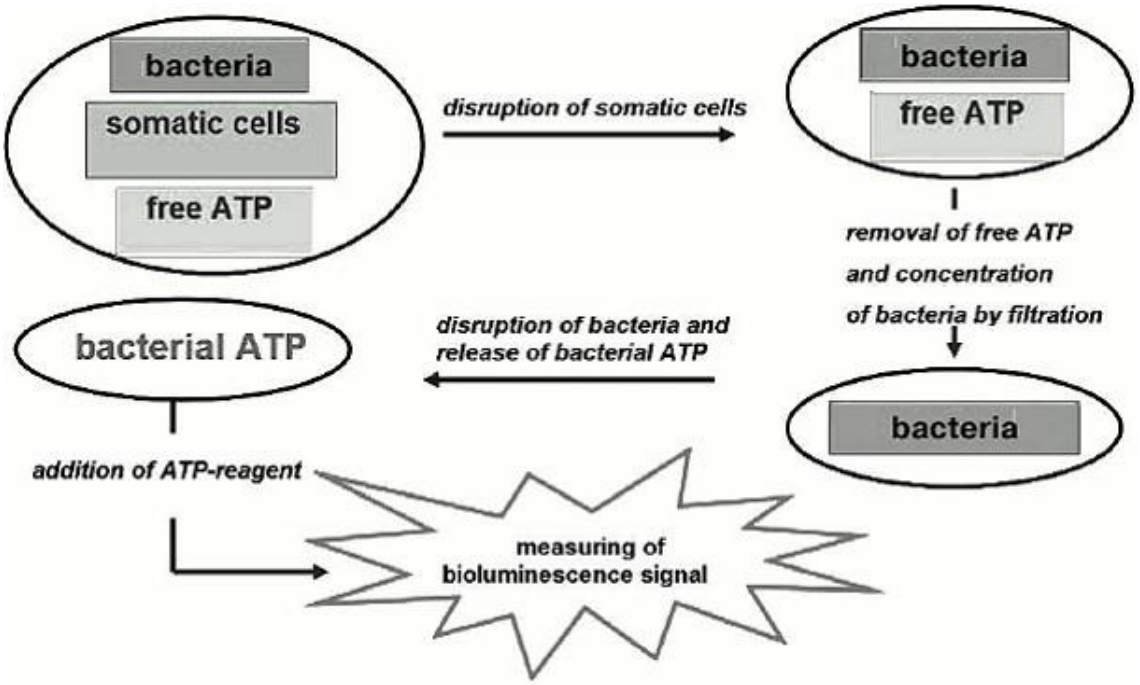

Figure 11. Schematic representation of bioluminescence determination mediated by ATP and enzyme luciferase as an indirect measure of cell viability[43]

The sensitivity and time-to-result of the ATP bioluminescence assay may also be improved with a two-phase reaction that begins by using an enzyme-catalyzed reaction to generate ATP to levels significantly higher than what is naturally contained in the microorganism. In the presence of microorganisms, specific microbial enzymes can be used to convert adenosine diphosphate (ADP) into ATP and adenosine monophosphate (AMP). The enzymes are not consumed by the reaction; therefore, ATP is continuously generated as long as ADP is present. The amplified ATP levels are then detected using the typical ATP bioluminescence reaction previously described [14].

The ATP+ method utilizes microbial enzymes and adenosine triphosphate (ATP) bioluminescent technology to rapidly assess the microbiological quality of pharmaceutical products using a presence/absence test method. The standard test includes a short growth-enrichment period, during which product is dispensed into standard microbiological culture medium and incubated for 24 hours. After incubation and sample preparation, an aliquot of the enrichment culture is transferred into a cuvette and placed into a luminometer. The traditional ATP bioluminescence assay is enhanced with linear, enzyme-mediated ATP amplification. Light output is measured by

the instrument's photomultiplier unit and recorded in Relative Light Units (RLUs). The results are compared to baseline RLU values and are classified as "Negative", "Positive", or "Overload" based on the signal-to-noise ratio. A negative result indicates that the product's bioburden level is below the assay's limit of detection whereas positive or overload results indicate that the product's bioburden level is at or above the test's limit of detection[44].

9.3.1.2 Non-Growth-based methods

Viability-based technologies use viability stains and laser excitation for the detection and quantification of microorganisms without the need for cellular growth. For this reason, organisms that are stressed, injured, in a dormant state or the previously mentioned viable but non-culturable state may be detected with these techniques, because they are metabolically active although they are not capable of growing in classical cultures. These types of technologies can be used for a variety of applications that require the detection and enumeration of microorganisms, such as bioburden and Microbial Limits testing, environmental monitoring, process water analysis and sterility testing[45,46].

1) Solid-phase cytometry

In solid-phase cytometry, basic principles of epifluorescence microscopy and flow-cytometry, which are fluorescent labelling of cells and laser detection are combined[45]. Equipment based in flow-cytometry has been developed in order for routine use in food and pharmaceutical companies, among others, and utilization of them require a series of steps: membrane filtration, fluorescence staining and scanning.

First, samples are filtered over a black membrane filter (e. g. polyester or polycarbonate) with an appropriate pore size (0.4 µm for bacteria and 0.8 – 2 µm for eukaryotic cells). These screen filters are used because of their low background fluorescence and high contrast, which facilitates validation using the epifluorescence microscope. Secondly, the retained cells are fluorescently stained using one of the different possibilities existing. General viability markers can be used to assess total viable count or specific genetic probes can be used to detect determined species.

Next, fluorescence emitted by the labelled cells is detected using a solid-phase cytometer. Generally, a laser beam excites the surface followed by a scanning device that detects fluorescence emitted in the green and amber spectra. Subsequently, the produced signals are processed by a computer to differentiate valid signals (labelled microorganisms) from fluorescent particles by evaluating data for several software parameters such as the size of the fluorescent spot, the specific intensity, the color ratio and the signal pattern[47,45].

In ChemScan, spots can be validated by personnel with the use of a fluorescence microscope, in case there is doubt of any result[48].

Another available option based on solid-phase cytometry is Sieve – ID ®, whose principle of operation is very similar to ScanRDI explained before although with some differences. Membranes used (Sieve) have been physically improved for a better laser-detection, made of silicon nitride and having an optically flat surface and a rigid plane. Argon-laser excitation has been replaced by a LED-based excitation system followed by automatic fluorescent detection. Scanner has been developed for giving an integrated image of 260 connecting scans of the sieve or membrane which is analyzed by an image processing software that can discriminate between fluorescently labelled cells or fluorescent particles on the basis of different parameters such as spot size, fluorescence, morphology, intensity, etc. similarly to ScanRDI. Spots can also be validated by instructed personnel, however, in the case of Sieve-ID the process is much more automatized as images with fluorescence detection are directly shown[49].

Solid-phase cytometry has different applications depending on the probe used. In the context of the pharmaceutical industry, it is a very useful tool for Total Viable Count, as fluorescent dyes can be specific for living cells. It is a quantitative technique, whose lower limit of quantification is 1 cell, so it could be applied to filterable samples such as water for pharmaceutical purposes or product bioburden of filterable solutions[48]. This technique is not growth-dependent so results can be obtained in a few hours, being a limiting factor the filtering capability of the sample.

Figure 12 shows a schematic representation of the solid-phase cytometry technique. As it is shown in the picture, only viable cells will emit fluorescence because they have been previously labelled with a viability specific marker.

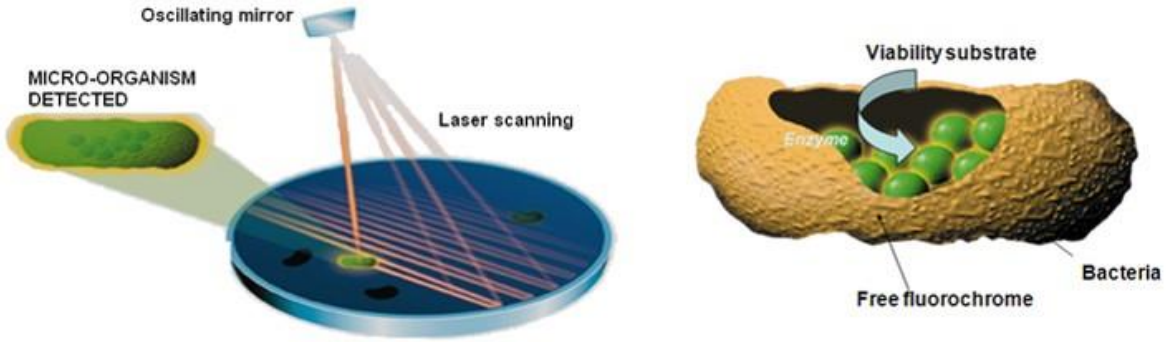

Figure 12. Schematic representation of solid-phase cytometry[50]

2) Optical spectroscopy and Light scattering/Intrinsic fluorescence

Spectrophotometric technology is capable of detecting viable particles distinguishing them from non-viable particles, being viable particles equivalent to living cells. Optical spectroscopy counting devices are designed to simultaneously detect the number and size of particles from a volume of air; and to additionally detect whether these particles are microbial and to estimate the number of microorganisms[51].

Optical spectroscopy methods utilize light scattering and other optical techniques to detect, enumerate and identify microorganisms without the need for microbial growth, labeling or amplification, and in many cases, obtain results in real-time.

These technologies present improved detection and accuracy. Because the detection of microorganisms is not reliant upon culture-based microbiological methods, the data relating to microbiological activity is arguably more representative of what is present within the clean room air. This limitation with culture-based techniques relates to the phenomena of viable but non-culturable state of microorganisms[35], already explained in this text.

Light scattering is a phenomenon in which the propagation of light is disturbed by its interaction with particles. Instrumentation that utilizes Mie scattering[52](i.e., where the scattered light intensity is dependent upon the particle size in a certain size range) and fluorescence detection techniques can provide information about the size and number of viable microorganisms in air. As microorganisms pass through a laser of a specific wavelength, certain metabolites, such as NADH, riboflavin and dipicolinic acid, are excited and provide an intrinsic fluorescent signal that distinguishes the microorganisms from other airborne particles. Therefore, these types of systems offer the simultaneous and instantaneous detection, sizing and counting of both viable and total particulates per volume of air.

Representative methods of this technology are for instance Bio-Vigilant and Biolaz, from Biomérieux and PMS respectively. Principle of operation is very similar in both methodologies.

The laser-induced fluorescence system detector's principle of operation is as follows: it continuously monitors the environmental air to measure the size of each individual airborne particle at a time scale of less than 1 millisecond (ms) and to concurrently determine whether that particle emits fluorescence or not. A threshold is set for the fluorescence signal. If the fluorescence signal is below the set level, the particle is marked inert. If the fluorescence signal exceeds the set level, the particle is marked biological. The combined data of particle size and fluorescence signal strength will determine the presence or absence of microbes on a particle-by-particle basis[53,54].

The laser-induced equipment is comprised of several instruments, which when integrated have the ability to enumerate microorganisms within a size detection range from 0.5 µm to $\geq$ 2 µm. The principle of operation of this alternative system is simultaneous measurements of individual particle size while measuring a UV-induced intrinsic fluorescence signal. Microbes contain certain metabolites necessary for life (i.e., NADH, riboflavin or dipicolinic acid) that, when excited at a certain wavelength, emit a known fluorescence signal that is captured by its sensing devices. This fluorescence signal detection serves as a marker for biological activity of the aerosol sample being processed. This marker is used to differentiate biologic particulates from inert dust particles. Model Biolaz®: air sampling flow rate equals 3.8 L/min; system has

a hard SS enclosure[53].

In the design illustrated in figure 13, the environmental air is drawn into the detector through a nozzle for particle sampling. The light source is a UV laser diode (wavelength 405 nm). The nozzle has an opening in its middle section to allow the laser beam to pass through perpendicular to the airborne particle stream. Directly downstream from the laser beam is the Mie scattering particle-sizing detector. Off axis from the laser beam, an elliptical mirror is placed at the particle-sampling region in such a way that the intersection of the incoming particle stream and the laser beam is at one of the two foci of the ellipsoid, while a Fluorescence Detector (in this case a photo-multiplier tube (PMT)) occupies the other focus. This design utilizes the fact that a point source of light emanating from one of the two foci of an ellipsoid will be focused onto the other. In this optical design, the Elliptical Mirror concentrates the fluorescence signal from microbes and focuses it onto the Fluorescence Detector. An optical filter is placed in front of the Fluorescence Detector to block the scattered UV light and pass the induced fluorescence. The data resulting from the microbial counts is displayed on the computer monitor as a count of the biologics present.

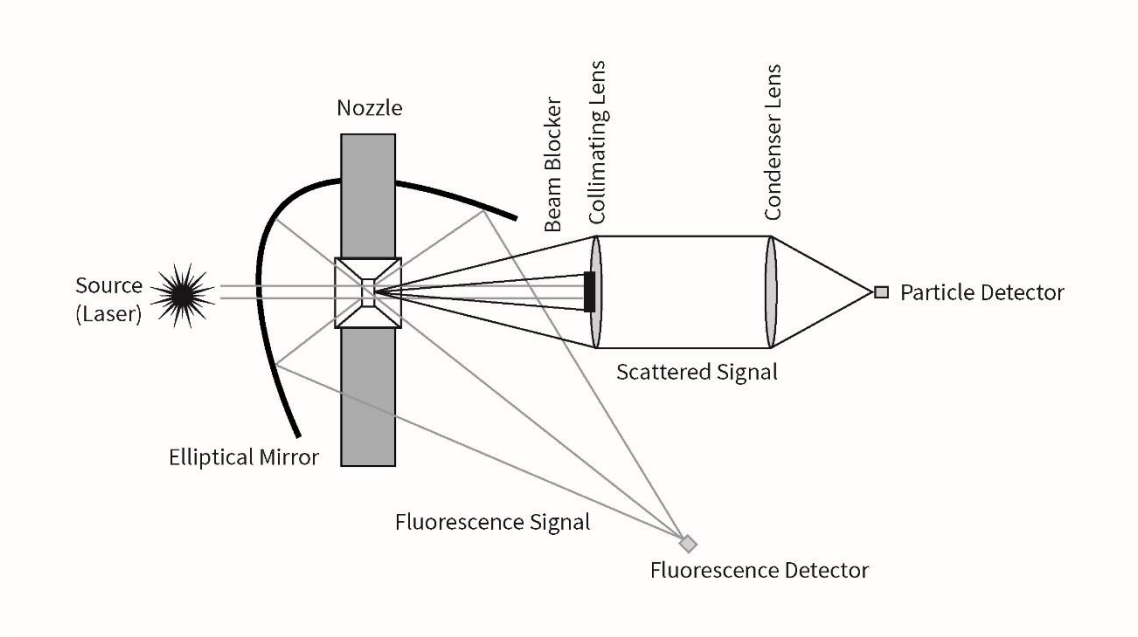

Figure 13. Schematic representation of optical spectroscopy technology for detection of viable cells in the air[53]

9.3.2 Microbial identification methods

9.3.2.1 Phenotypic Identification Methods

1) Utilization of Biochemical and Carbohydrate Substrates

There are a variety of technologies that employ microorganisms' ability to utilize carbohydrates as carbon source for the rapid and automated identification of microorganisms. A suspension of a pure culture (usually from an isolated colony on an agar plate) is inoculated onto test card, strips or well-plates, depending on the technique. Each recipient is composed of incubation wells, and individual wells contain a single substrate in dehydrated form. The inoculated cards, strips or wells are incubated, and if the microorganism under test utilizes substrates for cellular metabolism and growth, the turbidity, color and/or fluorescence in the well will change. The resulting data (normally in the form of positive or negative responses in each well) are compared with an internal database or reference library and a microbial identification (e.g., Genus and/or species) is provided. Some examples of equipment that utilize this technology are Vitek-2 or Biolog[55–57].

Figure 14 shows the workflow of identification of the system Omnilog. The plates on the left are the result of incubation of the cell culture with the dried substrates (one in each well). Purple dots mean positive growth. Plates are read in the visible spectrum and a pattern is obtained for data base comparison and identification.

Figure 14. Representation of Biolog workflow, which uses Omnilog technology based ob utilization of carbohydrate substrates for microbial identification[58]

2) *Fatty acid profiling*

The cellular membrane contains lipid biopolymers, and components of this cellular layer are fatty acid chains. Fatty acids can be extracted from a pure culture of microorganisms, and following a series of chemical conversion steps; the purified fatty acids are analyzed via automated gas chromatography (GC). The resulting gas chromatogram is compared with a previously established library of known microorganisms, and if a match is found, the identification is provided. This technique can be found in the market as MIDI Sherlock Microbial Identification system, however, this technique has not been well extended among pharmaceutical companies yet[59].

3) *Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry*

MALDI-TOF mass spectrometry provides an accurate molecular mass measurement and characterization of biomolecules, including proteins, peptides, polysaccharides and nucleic acids. Whole cells from an isolated colony are smeared onto a stainless steel plate and mixed with a UV-absorbing matrix. A laser ionizes the cells' biomolecules, which are then accelerated in an electric field. Within this field, the ionized molecules are separated according to their mass to charge ratio and the resulting mass profile is compared with an internal library of previously identified microorganisms.

Figure 15 shows how a MALDI-TOF system works in order to obtain microbial identification. A laser induces directly on the microbial colonies deposited, previously covered with an organic matrix. The organic matrix ionizes cellular components, mainly proteins and peptides, and the positively charged molecules travel along the electric field. Proteins and peptides are separated according to their mass/charge as it is directly related with the time of flight of each molecule.

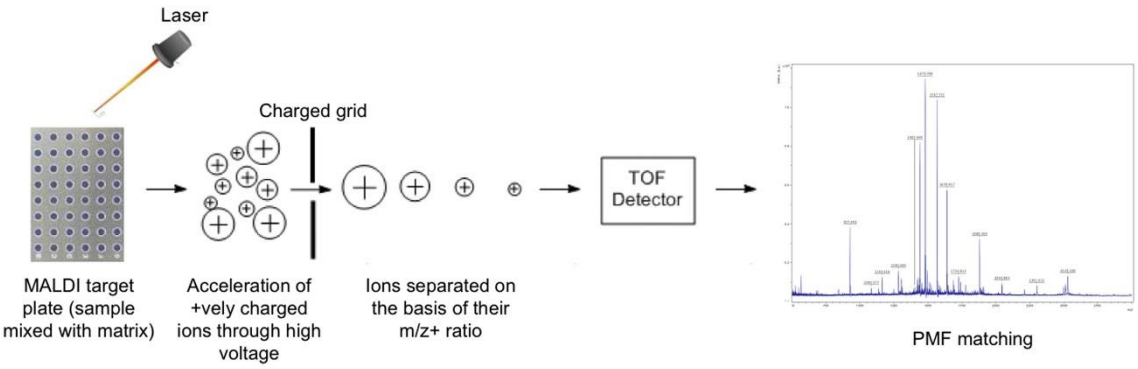

Figure 15. Representation of the mode of operation of a MALDI-TOF-based microbial identification system[60]

There are two main brands that have developed MALDI-TOF to a routine identification use, Biotyper (Bruker) and Vitek-MS (Biomérieux), each one having its own database. There aren't main differences between the performance of both methods[57,61,62]. Both systems have an IVD database for routine use that are validated and cannot be feed by the user. In terms of practical use, samples for Vitek-MS need slightly less preparation than samples for the Biotyper.

Figure 16 shows an example of a mass spectrum obtained of a bacterial isolate. The X axis represents the mass (m/z) in Daltons of each peptide separated during ionization. The Y axis represents the intensity of every peak. Background noise has been removed and only the most significant peaks are used for data base comparison. The combination of m/z forms the fingerprint of the isolate that will be used for identification.

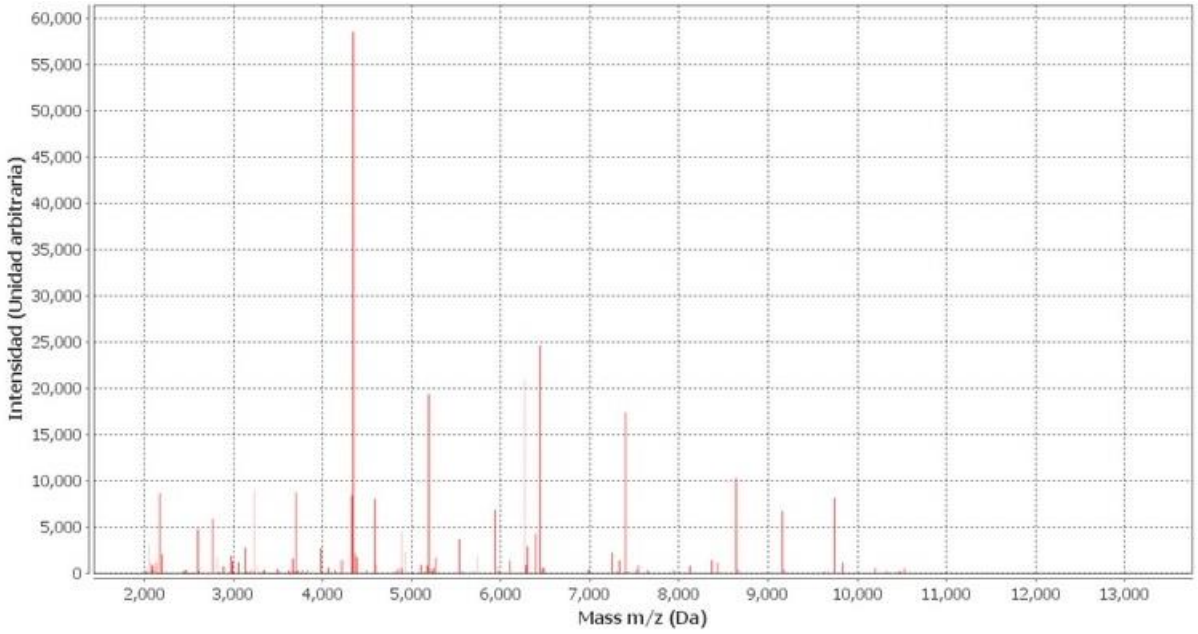

Figure 16. Example of a mass spectra fingerprint obtained from a bacterial isolate with Vitek-MS system

For each bacterial isolate, mass fingerprints are processed with the software algorithm which compares the characteristics of the spectrum obtained to those of the database, generating a final identification result. Spectral databases for the different systems are built by including different isolates of the same species that have been exposed to different time and culture medium during incubation, in addition isolates belonging to different geographical locations or from a different isolation year[63].

The algorithm calculates a percent probability, or confidence value, which represents the similarity in terms of presence/absence of specific peaks between the generated spectrum and the database spectra. Percentages or confidence values are distributed according to the following terms: 99.9% means a perfect match between the generated spectrum and the spectrum corresponding to an organism or group of organisms in the database; percentages between 60 and 99.8% mean a good and unique ID as the generated spectrum is sufficiently close to that of the database. Percentages lower than 60% are considered of low discrimination, it is possible that a unique profile is not found so percentage is divided into the number of probable ID[61,63,64].

9.3.2.2 Genotypic Identification Methods

Nucleic acid and gene amplification-based technologies employ a variety of scientific principles, including, but not limited to, DNA-based Polymerase Chain Reaction (PCR), RNA-based reverse-transcriptase amplification, *16S* rRNA typing, gene sequencing and other novel techniques. Many of these methods will detect the presence of a specific microorganism such as an "objectionable" or pharmacopeia "specified" organism, or can provide a microbial identification, in some instances, to strain subspecies level. Additional methods can be used to estimate the number of viable microorganisms in a sample, based on the number of amplification cycles required to reach a baseline threshold level. Nucleic-acid-based techniques have a wide variety of applications [65–67], although only a few examples of the most applied in pharmaceutical industry will be explained in this text.

There are two main types of genotypic methods: those based on genetic fingerprint and the ones based on DNA sequencing[68]. Genetic fingerprint methods typically use a systematic method to produce a series of fragments of an organism's chromosomal DNA, these fragments are then separated normally by gel electrophoresis, generating a profile or fingerprint that is specific of the given organism and its species. Data bases with fingerprints are created in order for samples to be compared to the patterns on the data base and determine which species it is[68].

In the case of sequence-based identification techniques, they rely on determining the nucleotide sequence of a specific stretch of DNA, usually although not always, associated with a specific gene. There are some regions in DNA that are susceptible candidates for identification as they have proven to have universally conserved regions at the beginning and at the end of the fragments while the remaining intermediate part of the sequence is sufficiently variable between species but conserved in between members of the same species.

Conserved regions at the beginning and at the end of the desired fragment are used for primers design, which are necessary for amplification of this fragment by PCR and for following sequencing of the fragment.

1) Ribotyping

To maintain correct RNA structure and ribosome function in bacteria, *16S* sequence of rRNA is highly conserved at the genus and species level, but there are nonconserved fragments within the rRNA operon that can be used for microbial identification and for strain differentiation. This method, known as ribotyping, uses restriction enzymes to cut DNA into fragments, which are then separated according to size and gel electrophoresis. The double-stranded DNA is then denatured to single-stranded DNA, which is subsequently hybridized with a rRNA operon probe and chemiluminescent agent. The resulting bands emitted by the fragments are compared with previously developed patterns from known microorganisms, and a bacterial identification is provided. Additionally, the differences observed within the same patterns can be used to provide information related to strain differentiation between bacteria belonging to the same genus and species[14].

2) Polymerase Chain Reaction and gene sequencing

One of the techniques employed for microorganism identification is amplification of a specific region of the organism genome, for example regions of *16S* or *18S* genes, followed by gene sequencing and comparison of the sequence obtained with a database of sequences, for example Nucleotide Collection or RefSeq in the National Center for Biotechnology Information (NCBI).

Figure 17 shows genetic regions present in both prokaryotic and eukaryotic cells that are commonly used for identification by sequencing. These genes are suitable for discrimination between species because their extreme regions are conserved (regions for primer design) but the middle regions present enough variability that allows species differentiation.

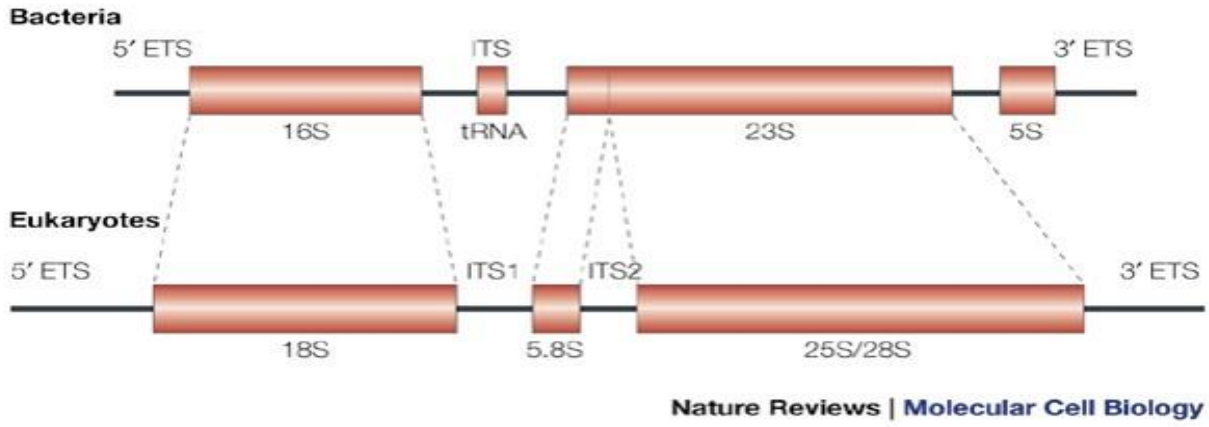

Figure 17. Schematic representation of genetic regions susceptible to be used for identification. Genes of both prokaryotic (bacteria) and eukaryotic (fungi) cells are shown[69]

In a classical PCR reaction, DNA is extracted from microorganisms (e.g. from an isolated colony on an agar plate) and heated to separate the double strands. DNA primers are then added which will bind to unique target sequences on the template DNA. The primer is elongated when the heat-stable DNA polymerase and nucleotide bases are added as reagents. The result is two new copies of the template DNA. This PCR process is then repeated, resulting in millions of copies of the target DNA. This step is necessary before gene sequencing in order to recover only the genetic sequence of the region of interest as well as increasing the quantity of DNA for sequencing reaction.

Gene sequencing is used for the identification of a wide variety of microorganisms, including bacteria, yeast and mold. The scientific principle involves sequencing each nucleotide base of a specific DNA sequencing after PCR amplification.

There are different sequencing methods, although the most used for bacterial identification is based on Sanger technique[70]. DNA is first extracted from a pure culture of cells and then amplified via PCR with a mixture of standard nucleotides and dideoxyribonucleotides. The latter ones, lack 3'-hydroxyl (-OH) group on their deoxyribose sugar. When a dideoxyribonucleotide is randomly incorporated during the amplification reaction, elongation of the PCR primer is terminated. This provides DNA fragments of varying lengths. Because each dideoxyribonucleotide is labeled with a different fluorescent dye, a series of fluorescently labeled copies of the amplified sequence, each terminating at a different base, is formed. These copies differ in

molecular weight and can be separated and detected (based on their fluorescence) using capillary electrophoresis of the reaction mixes. By simultaneously analyzing each of the four reaction mixes representing the four bases (A, T, C and G), software within the gene sequencer will reconstruct the linear arrangement of these bases in the sequence being analyzed. The resulting sequence is then compared with a library of known microorganism sequences, and if a sequence match is found, a genus and species identification is provided[14].

In the Identification of microorganisms by sequencing, several genes have been described, some of them specific for distinguishing bacterial genus or species[71]. Regarding pharmaceutical industry, sequencing of universal genes is preferred as different types of bacteria can be found.

Traditionally, sequence-based methods have proven more effectiveness than genetic fingerprinting in establishing a broad phylogenetic relationships among bacteria[68]. Several genes have been described for phylogeny studies in bacteria, although the most used until now is *16S* ribosomal unit gene[72]. In the case of fungi, it is more difficult to find a universal gene such as *16S* in bacteria[73] in part because data bases containing fungal sequences are not as wide as those of bacteria. However, several genetic regions have been long studied and described for fungal genetic identification.

In the pharmaceutical industry, according to Pharmacopoeia, not always identification to the species level is necessary. However, there are specific cases such as positives in sterility tests or media fill contamination, among others, that require molecular identification. In addition, in the case of investigation in microbiology, identification is necessary and can provide very useful information on the origin of contamination. For that reason, although phenotypic methods may be faster than genotypic ones, the last ones are more reliable and accurate in the case of species identification.

9.3.2.2.1 Gene sequencing of Bacteria

As it has been mentioned, the most commonly used region for bacteria identification by sequencing is *16S* gene, which encodes for the small subunit (SSU) rRNA of the bacterial ribosome. This gene has about 1500 bp and has interspecies variability although it is

conserved intraspecies, acquiring the grade of universal gene. As it is one of the most studied regions of bacterial DNA for phylogenetic diversity determination, existing sequences on the data bases are a great amount, so the value of this gene as universal region increases with time as the data base grow[74]. Identification can be performed either by sequencing the first 500 base pairs of the *16S* rRNA or the entire gene sequencing in order to obtain greater accuracy.

An essential contribution to the utility of this gene is the study of the more- and less-conserved regions of its sequence, in order to find potential candidate segments for primer binding. This allows specific amplification of the region desired in all bacterial metagenome. Different primer pairs have been designed in order to study *16S* gene, primers that sequence the first 500 bases of the gene have been widely used. However, the whole sequence of the gene can also be used for identification and data base comparison. When comparing the sequence to the data base, the use of a longer sequence may provoke less similarity score although the identification will be more accurate.

Figure 18 shows the different conserved and variable regions of gene *16S* from bacteria. Primer positions used for sequencing and identification are represented. Blue segments represent conserved regions to which primers are specific for and primers are represented as green (forward) and orange (reverse). Red segments represent variable regions whose sequence is different between species and determined the identification.

Figure 18. Schematic representation of *16S* gene of bacteria with conserved and variable regions[113]

9.3.2.2.2 Gene sequencing of Fungi

In contrast, different regions of the fungal genome have been described for broad analysis of fungal phylogenetic diversity. For example, nuclear large subunit (LSU) *25S* or *28S* and *18S* small subunit (SSU) of the ribosome. These genes form part of a tandemly repeat cluster that also contains the *5.8S* rRNA gene with transcribed and non-transcribed spacer regions. They have been used predominantly in phylogenetic studies to determine evolutionary relationships between taxa, and these sequences provide a very useful tool for identifying environmentally amplified rDNA[73].

Different genetic variations have been observed for these genetic region. The nuclear small subunit rRNA gene is the most conserved among family level and resolves little phylogenetically[75]. The large subunit rRNA gene is more variable and resolves well phylogenetic variations between genus levels[76]. In order to discriminate fungal species, more information is needed, for that the internal transcribed region (ITS) displays the greatest sequence and size variation for this gene cluster[73]. However, many species contain variations between its members in this region.

Figure 19 shows how the gene cluster fort the nuclear ribosomal RNA studied in fungal phylogeny is distributed. The *18S* sequence has diverse variable regions, shown in black bars in the V4, D2 and D8 segments in the figure, while conserved regions are represented by the white bars. Different primer combinations have been used in order to identify fungi although the primer pair which covers most part of the sequence gives a higher resolution[77].

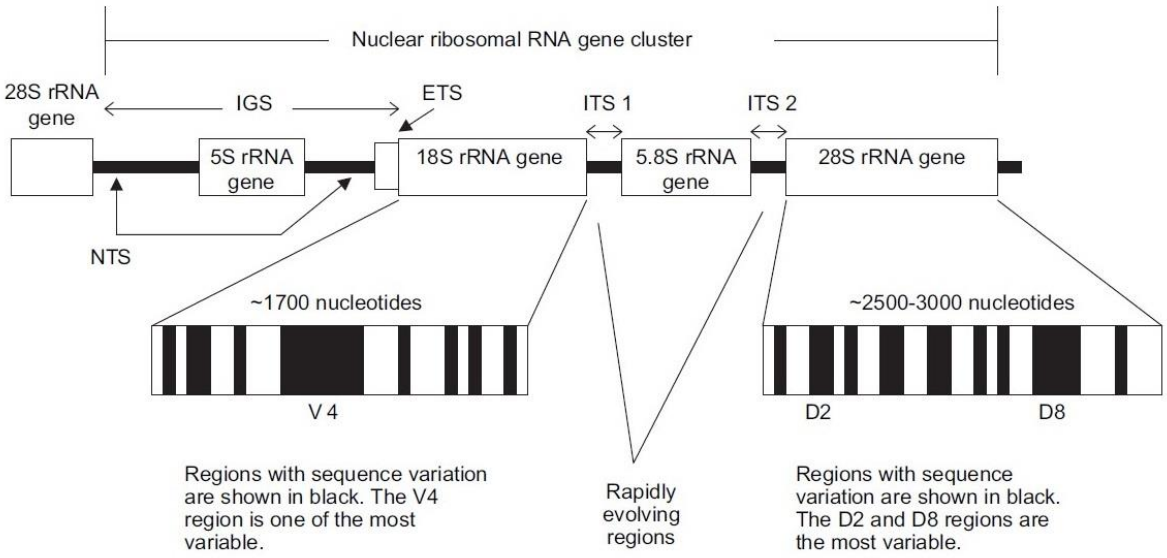

Figure 19. Schematic representation of nuclear ribosomal RNA gene cluster showing variable and conserved regions of *18S* gene[73]

9.4 Future and regulatory perspectives of rapid methods

Although implementation of rapid microbiology methods in the pharmaceutical industry is being a tough task, regulation encourages its application in different regions. As said before, regulators will accept any change in manufacturing or testing process that has proven to be equivalent, superior to, and/or non-inferior than the system currently in place[14].

FDA has probably been the most open in the use and application of rapid methods in microbiology. In its *2004 Guidance for industry: Sterile Drug Products Produced by Aseptic Processing* appears a recommendation in the use of genotypic methods for investigation of contamination, as they have shown higher accuracy than biochemical and phenotypic techniques[26]. In the case of cellular and gene therapy products, FDA encourages the use of rapid microbiological methods for sterility testing of these products. Justification for this is that latter advances in technology in the recent years have allowed the development of new sterility test methods that yield accurate and reliable test results in less time and with less operator intervention than the currently prescribed methods[78,79].

In every case, it is easier to apply rapid methods to new drug applications (NDA) as they still need marketing authorization, rather than changing the existing dossiers of drugs already in the market.

In the case of European regulatory agency, use and application of rapid methods is also encouraged. EMA suggests the utilization of its Scientific Advice procedure and dialog with regulators in order to justify any post approval changes. In addition, European Pharmacopoeia includes a chapter on validation of alternative methods in microbiology, as well as their American counterparts[80].

Japan and Australia have their own Pharmacopoeia or generally British, European or United States Pharmacopoeia are used as reference.

Expectation from regulators is that rapid methods will be implemented and embraced by every time more pharmaceutical industries, as advancement in technology has a positive consequence on the quality of products.

Materials and Methods

The materials and methodologies used for this project development are described in the following lines.

1 Identification of microorganisms

1.1 Microbial strains used in validation of Vitek-MS and PCR and sequencing methods

Validation of Vitek-MS and PCR-Sequencing methods were performed using reference strains. Table 3 shows the strains from the American Culture Type Collection that were used in both validations.

Table 3. ATCC strains used in validation of Vitek-MS and PCR-Sequencing

Method	Strain
Vitek-MS	*Candida albicans* ATCC 10231
Vitek-MS	*Clostridium sporogenes* ATCC 19404
Vitek-MS/PCR-Sequencing	*Staphylococcus aureus* ATCC 6538
Vitek-MS/PCR-Sequencing	*Bacillus subtilis* ATCC 6633
Vitek-MS/PCR-Sequencing	*Pseudomonas aeruginosa* ATCC 9027
Vitek-MS/PCR-Sequencing	*Escherichia coli* ATCC 8739
Vitek-MS/PCR-Sequencing	*Micrococcus luteus* ATCC 10240
Vitek-MS	*Saccharomyces cerevisiae* ATCC 9763
Vitek-MS/PCR-Sequencing	*Kocuria rhizophila* ATCC 9341
Vitek-MS	*Aspergillus brasiliensis* ATCC 16404
Vitek-MS/PCR-Sequencing	*Enterococcus hirae* ATCC 10541
Vitek-MS/PCR-Sequencing	*Staphylococcus epidermidis* ATCC 12228

In the validation of PCR-Sequencing, apart from ATCC strains, internal ones identified by a previously validated method were used. Table 4 shows the internal strains used for validation of PCR-Sequencing method and how they were identified.

Table 4. Strains used in validation from standardized internal common isolates or previously identified by Vitek-MS

Internal strains used in qualification	
Strain	**Identification method**
***Burkholderia cepacia* (Ref: 001_SJD_BURK.CEPAC)**	MALDI Biotyper Bruker
***Staphylococcus epidermidis* (Ref: 002_SJD_STAP.EPIDE)**	MALDI Biotyper Bruker
Staphylococcus haemolyticus	Vitek-MS
Staphylococcus hominis	Vitek-MS
Kytococcus schroeteri	Vitek-MS
Kocuria palustris	Vitek-MS

1.2 Traditional methods

1.2.1 Culture media

Different culture media were used to promote microbial growth. Generally, bacteria were grown in Trypticase Soy Agar (TSA) (Scharlab Ref: 064-PA0031) for PCR-Sequencing method. In case there was any difficulty in recovery, such as waterborne bacteria, Reasoner's 2A Agar (R2A) was used (Scharlab Ref: 064-PA0072I). In order to identify bacteria by Vitek-MS method, Columbia Blood Agar (Scharlab Ref: 064-PA0004) was used.

Fungal growth was performed using different type of culture media. For macroscopical and microscopical morphology observation, potato dextrose agar (PDA, Scharlab Ref: 01-483) was used. To grow fungi for Vitek-MS or PCR-Sequencing identification Saboureaud 4% Dextrose Agar (Scharlab Ref: 064-PA0147) was used.

1.2.2 Incubation conditions

Incubation conditions for microbial growth were different for bacteria and fungi. Bacteria were incubated at 32.5 ± 2.5°C for at least 24 hours, until enough cellular mass was achieved. Fungi were incubated at 22.5 ± 2.5°C for at least 48 hours, until enough cellular mass for identification was achieved.

1.2.3 Microbial staining and microscopy observation

- Gram staining of bacteria

  Colonies were picked from the plate culture and spread in a NaCl 0.9% drop in a glass slide. The drop was dried with heat and submerged in gentian violet (hexamethyl pararosaniline chloride 5.0 g/l in water), Gram's iodine solution (0.33% Iodine and 0.66% potassium iodine) and basic fuchsine (0.1% w/v) sequentially, washing with water after every stain and ethanol after iodide solution. When the slide is dry, cells are observed in an optical microscope at 100x with immersion oil.

- Methylene blue staining for yeast

  Single colonies from plate cultures were picked and spread with a water drop on a glass slide. The drop was dried with heat and then submerged in Methylene blue solution (0.1% w/v) for approximately 3 minutes. A washing step with water is performed to remove residual staining and once the slide is dry cells are observed in an optical microscope at 100x with immersion oil.

- Microscope observation of molds

  A fragment of the mold culture the size of the loop is spread in a lactophenol cotton blue (0.1% w/v) drop on a glass slide. The drop is covered and fungal structures are observed freshly with an optical microscope at 40x.

1.2.4 Morphological identification of fungi

Filamentous fungi were cultured in Potato Dextrose Agar (PDA) using 3 point seeding in order to obtain individual colony. Morphology was assessed observing color (upside and downside), texture, diffusible pigments, exudates, growth zones, aerial and submerged hyphae and colony topography.

1.2.5 Cryopreservation system Protect®

Protect ® system is a low temperature long-term storage method for preservation of microorganisms. Tubes were prepared with beads in the storage solution within the tube. The isolates to be preserved were grown in a solid culture media plate until enough cellular mass was recovered and suspended in the tube. Afterwards, tubes were vortexed and centrifuged (12000 rpm during 1 minute), the remaining liquid in the supernatant was removed and the tubes stored at -80 ±5°C. In order to recover the isolate for culturing, a bead was spread with a sterile loop in a culture media plate or suspended in liquid media.

1.3 Biochemical Identification Method MALDI-TOF

1.3.1 Sample preparation

Colonies were picked from plates with a 1-µl plastic loop, spotted onto disposable target plates in duplicate, and 1 µl of the matrix solution (α-cyano-4-hydroxycinnamic acid, VITEK® MS CHCA) was added to both smears. The yeast isolates were prepared for MS analysis using a direct, on-target, extraction method with a 1-µL formic acid overlay that was allowed to dry before matrix application. *E. coli* ATCC 8739 was used to calibrate and control the method according to the manufacturer's instructions.

1.3.2 Mass spectra generation and result

Mass spectra are generated with a mass spectrometer (Vitek-MS, Biomérieux) in positive linear mode at a laser frequency of 50 Hz with an acceleration voltage of 20 kV and an extraction delay time of 200 ns. For each spectrum, 500 shots in 5-shots steps from different positions of the target spot (automatic mode) are collected by the mass spectrometer, generating a mass fingerprint with the most abundant peaks for each isolate. Measured mass spectra ranged from 2,000 to 20,000 Da.

The spectra were analyzed by bin matrix on the VITEK® MS v2.0 IVD database. In this process, a weight is assigned to each peak specifically for each species and 1300 bins or peak areas are analyzed. A green square, yellow triangle, or red circle was assigned to each identification to reflect high, medium, or low confidence, respectively.

1.4 Molecular Identification Method: PCR and Sequencing

1.4.1 DNA extraction

DNA extraction was performed from plate culture of the isolates. DNA extraction protocol was slightly different for bacteria and fungi:

Bacterial cells were suspended in 1 ml 1X TE (10 mM Tris-HCl ph 7.5 and 1 mM EDTA pH 8.0) and centrifuged during 1 minute 12000g to obtain a pellet for lysis in a 1.5 ml Eppendorf tube. Pellet was resuspended in 180 µl of lysis solution and incubated during

1 hour at 37°C. Bacterial lysis solution contained 20 mM Tris pH 8.0; 2 mM EDTA pH 8.0; 1.2% Triton X-100; 20 mg/ml Lysozyme (Roche Ref: 10837059001); 0.2 mg/ml Lysostaphine (Sigma Ref: L7386-5MG). After that, 20 µl of Proteinase K and 200 µl of AL Buffer (both from QIAamp DNA Mini Kit, Qiagen) were added and the solution was incubated during 30 min at 55°C.

Filamentous fungal cells were suspended in 1 ml TE 1X and centrifuged for 1 minute 12000g. The supernatant was discarded and the pellet was incubated during 10 minutes at 95°C in 200 µl of 50 mM NaOH solution. Then, cells were centrifuged during 5 minutes 10000g, the supernatant was discarded and the pellet was resuspended in 500 µl of lysis solution. Fungal lysis solution contained 10 UI/ml lyticase (Sigma Ref: L2524), 50 mM Tris pH 7.5, 10 mM EDTA and 28 mM β-Mercaptoetanol. Cells were incubated with lysis solution for 1 hour at 37°C. After that, the solution was centrifuged during 10 minutes at full speed, the supernatant was discarded and the pellet was resuspended in 180 µl of ATL buffer and 40 µl Proteinase K (both from QIAamp DNA Mini Kit, Qiagen). Cells were incubated during 30 minutes at 55°C.

Both bacterial and fungal DNA extraction protocols followed with 200 µl addition of absolute ethanol and the following steps were performed according to QIAamp DNA Mini Kit (Qiagen) handbook.

The size of the genomic DNA was checked by loading 1 µl of genomic DNA with 9 µl of loading buffer in a 0.7% agarose gel, using lambdaHind III as molecular marker. Quantity of DNA in ng/µl was assessed with Nanodrop Lite (wavelength 260-280 nm).

1.4.2 PCR conditions

PCR amplifications of the *16S* and *18S* genes for bacteria and fungi respectively were performed in two 50 µl reaction for each sample, in order to optimize results. Table 5 shows the sequence of primers used for DNA amplification of bacteria and fungi as well as the gene region they are specific for.

Each reaction contained 5 µl of 10X Expand High Fidelity Buffer with 1.5 mM MgCl2 (Roche ref: 11 759 078 001), 200 µM of each dNTP (VWR Ref: 733-1364), 2.5 U Expand High Fidelity Enzyme Mix (Roche ref: 11 759 078 001) and 0.5 µM of each primer, with

the appropriate combination for bacteria and fungi. An amount of 100 ng of DNA per reaction was calculated.

PCR conditions for amplification of *16S* gene of bacteria were: 5 min at 95°C, then 30 cycles of 30 seconds of denaturing at 95°C, 30 seconds of primer annealing at 53°C and 1 minute and 30 seconds of extension at 72°C; finally an extension step of 7 minutes at 72°C.

PCR conditions for amplification of *18S* gene of fungi were: 8 min at 95°C, then 35 cycles of 30 seconds of denaturing at 95°C, 45 seconds of primer annealing at 47°C and 3 minutes of extension at 72°C; finally an extension step of 10 minutes at 72°C.

The size of the PCR size (around 1.4 kB) was checked by loading 5 µl of PCR product with 5 µl of loading buffer in a 1% agarose gel, using DirecLoad™ (Sigma Ref: D3937-1VL) as molecular marker. Quantity of DNA in ng/µl was assessed with Nanodrop Lite (wavelength 260-280 nm).

1.4.3 Sequencing and BLAST search

Sequencing was performed in Microsynth services (Microsynth AG). *16S* sequence of bacteria was obtained with 4 different reactions of primers 27f, 1492Rv, 786f and 786Rv, then the contig sequence was obtained with Chromas Pro software version 2.0.1. 18S sequence of fungi was obtained with 2 reactions of primers also used for PCR FR1 and NS1, the contig sequence was also obtained with Chromas Pro software version 2.0.1. The list of primers with their respective sequence is shown in table 5.

Contig sequence of every isolate was searched with nucleotide BLAST tool (National Center for Biotechnology Information, NCBI). The databases used for sequence comparison were *16S* ribosomal RNA sequences (Bacteria and Archaea) for bacterial identification and RefSeq genome Database for fungal identification

Table 5. List of primers used for PCR and sequencing

Primer Name	Nucleotide Sequence 5' – 3'	Use
27f	AGAGTTTGATCCTGGCTCAG	*16S* gene Bacteria (PCR and Sequencing)
1492Rv	GGTTACCTTGTTACGACTT	*16S* gene Bacteria (PCR and Sequencing)
786f	GATTAGATACCCTGGTAG	*16S* gene Bacteria (Sequencing)
786Rv	CTACCAGGGTATCTAATC	*16S* gene Bacteria (Sequencing)
FR1 [1]	AICCATTCAATCGGTAIT	*18S* gene Fungi (PCR and Sequencing)
NS1	GTAGTCATATGCTTGTCTC	*18S* gene Fungi (PCR and Sequencing)

[1] FR1 has two inosine modifications in order to enhance primer annealing.

2 Laser-induced fluorescence system for environmental air monitoring

2.1 Traditional methods used in environmental air

Traditional methods to detect microbes in air consist of active air sampling and passive air sampling methodologies. For qualification purposes, laser-induced fluorescence method was compared only to active air sampling. However, in routine, laser-induced fluorescence system worked in parallel with both active air and passive sampling.

2.1.1 Active-air sampling SAMPL'AIR

The air sampler used in the comparison was SAMPL'AIR™ Pro from bioMérieux, which sampled air at a flowrate of 100 L/min that impacts on an agar plate. Air was sampled by both the SAMPL'AIR for 10 minutes to achieve a total sampling volume of 1000 L (1 m^3) in four classes of cleanrooms: A, B, C and D. Collected air impacted on an agar plate (TSA) which was incubated for two days at 30°C and then two additional days at 20°C to promote growth of both bacteria and fungi. Plate counts for colonies were performed manually.

2.1.2 Passive air sampling settle plates

Sampling by settle plates during the 6-months evaluation period was performed by locating an open culture media plate in the appropriate location (locations defined on the basis of a risk analysis, data not shown). Settle plates were used during aseptic filling and changed every 4 hours if the activity monitored was longer than this time. Plates were incubated for two days at 30°C and then two additional days at 20°C to promote growth of both bacteria and fungi. Plate counts for colonies were performed manually.

2.2 Alternative method Laser-Induced Fluorescence System Technology

Ten measures of a discrete air volume were taken for "at rest" and "in operation" conditions in four classes of cleanrooms: A, B, C and D. The volume of air sampled with the alternative system was adjusted according to the particle content limit of each room. Biolaz® had a flowrate (3.8 L/min), considerably lower than that of Sampl'air. A longer time period was necessary for the alternative system to record the same volume of air as the traditional system. So, in Class A, a volume of 1 m^3 was needed, however, as microbial count was higher in areas B, C and D, 500 L were collected in room class B, 200 L in room Class C and 100 L were collected in room Class D. The summary of sampling zones along with the volume of air taken and the collection time for the alternative method are shown in table 6.

Table 6. Summary of sampling zones and samples taken in every room during laser-induced fluorescence system qualification

Room Class	Activity Performed in the room	Volume of air collected	Collection time
A	Aseptic filling	1000 L	5 hours
B	Changing room to sterile zone entry	500 L	2.5 hours
C	Manufacturing zone	200 L	1 hour
D	Cell culture laboratory	100 L	30 min

2.3 Statistical approach

2.3.1 Comparison of traditional and alternative methods

To compare the performance of the alternative system to the traditional active air sampling method, both methods were used simultaneously in the same area and measured equivalent volumes of air. According to PDA Technical Report No. 33 (Revised 2013)[14], agreement among individual results is demonstrated when the procedure is applied to every sampling of the same suspension using different suspensions across the test range. In this case, we used the different microbial air suspensions present in each classification of cleanroom.

An equivalence test was used to assess the statistical equivalence between population mean of the laser-induced fluorescence system and the active air sampling in plate method, using Minitab 17 software. Equivalence test compares the two population means using the following hypotheses structure:

*Null Hypothesis _H*0_:

$\mu Biolaz - \mu Sampl'air \leq$ Lower Bound

$\mu Biolaz - \mu Sampl'air \geq$ Upper Bound

*Alternative Hypothesis _Ha_*:

Lower Bound $\leq \mu Biolaz - \mu Sampl'air \leq$ Upper Bound

In this approach, the null hypothesis assumes the two population means are different by at least a pre-defined threshold amount. If the confidence interval is entirely within the lower and upper bounds, the two population means are declared comparable. If the confidence interval is not entirely within the bounds, the two population means are not declared comparable and the null hypothesis remains in effect stating that the two population means are different.

2.3.2 Calculations for setting alert and action limits

Alert and action limits for the alternative method was determined for Class A according to PDA Technical Report No. 13 *Fundamentals of an Environmental Monitoring Program*[13] and TR No. 59 *Utilization of Statistical Methods for Production Monitoring*[81],

following the *Non-parametric Tolerance Limit approach*, as the vast majority of values obtained were skewed towards 0:

- Alert level: with a 0.95 significance including 95% of the data population.
- Action level: with a 0.95 significance including 99% of the data population.

Minitab 17 was used for nonparametric statistical approach.

3 Solid-phase cytometry based system for quantification of microorganisms

3.1 Samples used

Different samples were used in the different tests carried out with MuScan. Sterile water and phosphate buffer solution (PBS) were commercial and used as negative controls while the other samples were taken from RJF facilities and were used as test samples.

- **Sterile water**. Distilled water from Gibco (Ref: 15230162), normally used in cell culture, was used as water with no microbial burden.
- **PBS**. Sterile PBS from Gibco (Ref: 20012027) was used as negative control in some of the experiments.
- **Water for Injection (WFI)**. WFI was collected from a 80°C point of use and packed in sterilized borosilicate glass bottles for shipment.
- **Vancomycin**. Vancomycin 1g lyophilized was used for testing purposes.
- **Ear spray**. A sample of one aerosol bottle of a saline-based ear washing spray was used for testing purposes.

3.2 Microorganisms used for spiking

Spiking experiments were performed using Bioballs for microbial inoculum. Two different species were used: *Pseudomonas aeruginosa (NTC12924)* and *Candida albicans (NCPF 3179)*.

In addition, an overnight culture of *Pseudomonas aeruginosa* from laboratory use was added to vancomycin for the spiking experiment, instead of bioballs. Cells in the

overnight culture were quantified with MuScan in order to adjust cell quantity for the experiment.

3.3 Equipment

The solid-phase cytometry system consists of different components: a filtration system, a fluorescence scanner and the software that analyzed the scans containing fluorescence.

- Milliflex filtration system. Milliflex pump for filtration was used both with the traditional and the MuScan systems. The pump is connected to vacuum in order to force filtration of the samples through the Milliflex membrane or the sieve for the MuScan.

- MuScan detection device. Scanning is performed by a LED-based optical (fluorescent) scanning read out of the surface of the microsieve. Before each scan, the focus plane is determined, ensuring in focus scanning of the whole microsieve membrane surface. An integrating image of approximately 260 connecting scans of the microsieve surface assembled and analyzed in a step-and-scan procedure.

- Software. In total viable count, the software automatically gives a number of cells in base of the fluorescence detected. Different parameters such as size, shape or intensity are adjusted for fluorescence to be detected as cells.

3.4 Procedure

Every assay was performed in parallel with traditional method and the alternative method.

3.4.1 Traditional method Milliflex filtration and plate count

Liquid samples were filtered using Milliflex filtration system. Milliflex filtration system consists of membrane cassettes (0.45 µm pore size Ref: RMHVMFX24) and a vacuum

pump. The cassettes were put over the vacuum pump that forces the liquid to pass through the membrane. Then, membranes were put on a TSA plate and incubated at 30ºC for 3 days. Microorganisms that were retained by the membrane grew on the TSA plate. Plate count was performed every 24 hours to check number of CFU.

3.4.2 Alternative method solid-phase cytometry MuScan

Milliflex vacuum pump was also used for filtration of samples through MuScan sieves. Liquid samples passed through the sieve so all microorganisms present in them were retained. Then, the sieve was washed with buffers A, B and C (Sieve-ID® Total Viable Count Kit, from Innosieve Diagnostics Ref: ID-201001-10). Washing steps were performed with the help of a vacuum pump. A specific viability dye (not specified) was then added and microsieve was incubated for at least 30 minutes in the dark. Finally, residual dye was removed by two steps of buffer washing and centrifugation (1 minute at maximum speed). Once the microsive was dry, it was introduced in the scanning device for fluorescence reading.

Results

1 Validation and implementation of microbial identification methods in both the microbial control and the monitoring of pharmaceutical manufacturing

1.1 Validation of phenotypic and genotypic identification methods

Identification of microbial isolates in the pharmaceutical manufacturing environment and products gives useful information on the origin of a contamination. In addition, it is important to know which the most frequently found species are in order to prevent contamination.

Microbial monitoring of pharmaceutical environments and products involves different activities in which microorganisms must be isolated. Besides, it is important to establish an identification program to fix which isolates need further characterization and what level is required.

Implementation of new methodologies in a pharmaceutical company requires validation of the technique according to cGMPs. For that, identification methods of the program were validated before its implementation. Specifically, Vitek-MS and PCR-Sequencing methods were chosen as phenotypic and genotypic methods, respectively.

1.1.1 Acceptance criteria for validation of microbial identification methods

Validation of microbial identification methods was performed according to EP and USP. Both EP and USP have specific chapters on verification of microbial identification methods[80,82].

The approach followed for validation was testing 12 strains from the American Culture Type Collection (ATCC) by Vitek-MS and 8 ATCC strains and 4 in-house strains by PCR-Sequencing.

Validation parameters evaluated were accuracy, precision and reproducibility. These parameters are defined as following:

- Accuracy: assesses closeness of the result to the reference microorganism. Accuracy is calculated as the number of results that coincide with their reference strain divided by the number of the total identifications.
- Precision: assesses that the system is repetitive under the same conditions. Precision is calculated as the number of correct identifications divided by the total number of identifications performed. Ten identification of the same isolate are performed in order to prove that the result is repetitive.
- Reproducibility: assesses that the system is repetitive under different conditions, such as different days or different analysts.

A percentage higher than ninety was accepted for all parameters and individual results were considered valid if percentage of confidence was higher than 90%.

1.1.2 Validation of Vitek-MS

1.1.2.1 Accuracy of Vitek-MS

Accuracy was assessed by performing the identification of 12 ATCC isolates with Vitek-MS in duplicate. Results obtained are summarized in table 7.

Regarding identifications obtained for each sample in table 7, all results from Vitek-MS coincide with their ATCC certificates. The percentage of confidence given for each result is higher than 99%. Both replicates of *Bacillus subtilis* were identified as a mixture of 3 species: *B. vallismortis, B. subtilis* and *B. amyloliquefaciens*, so only genus can be determined with a percentage of confidence higher than 90%.

Regarding the results obtained, Vitek-MS is accurate for the identification of microorganisms.

Table 7. Assessment of the accuracy in Vitek-MS method

Strain of reference	Replicate 1 [a]		Replicate 2 [a]	
	ID Result [b]	% Conf.[c]	ID Result[b]	% Conf.[c]
***S. epidermidis* ATCC 12228**	+	99.9	+	99.9
***S. aureus* ATCC 6538**	+	99.9	+	99.9
***M. luteus* ATCC 10240**	+	99.9	+	99.9
***E. coli* ATCC 8739**	+	99.9	+	99.9
***E. hirae* ATCC 10541**	+	99.9	+	99.9
***K. rhizophila* ATCC 9341**	+	99.9	+	99.9
***B. subtilis* ATCC 6633**	*Bacillus vallismortis*	33.3	*Bacillus vallismortis*	33.3
	Bacillus subtilis	33.3	*Bacillus subtilis*	33.3
	Bacillus amyloliquefaciens	33.3	*Bacillus amyloliquefaciens*	33.3
***P. aeruginosa* ATCC 9027**	+	99.9	+	99.7
***C. sporogenes* ATCC 19404**	+	99.9	+	99.9
***C. albicans* ATCC 10231**	+	99.9	+	99.9
***S. cerevisiae* ATCC 9763**	+	99.9	+	99.9
***A. brasiliensis* ATCC 16404**	+	99.9	+	99.9

[a]Two replicates were assayed for each strain.
[b] +, the identification is coincident with the strain of reference.
[c]The criteria for accepting the results was a percentage of confidence higher than 90 %.

The mass profile obtained with Vitek-MS of one replicate of the *Bacillus subilis* ATCC6633 is shown in figure 20 in which percentage of confidence of the three results are shown. Partial sequences of *16S* gene from *B. vallismortis, B. subtilis* and *B. amyloliquefaciens* were retrieved from the National Centre of Biotechnology Information (NCBI) to align them and search for differences at nucleotide level. Accession number of *B. vallismortis, B. subtilis* and *B. amyloliquefaciens* sequences were EF433404, NR_112116 and NR_116022 respectively. Regions of the alignment in which differences were detected are shown in figure 21.

Figure 20. Mass profile obtained with Vitek-MS for *Bacillus subtilis* ATCC6633 strain

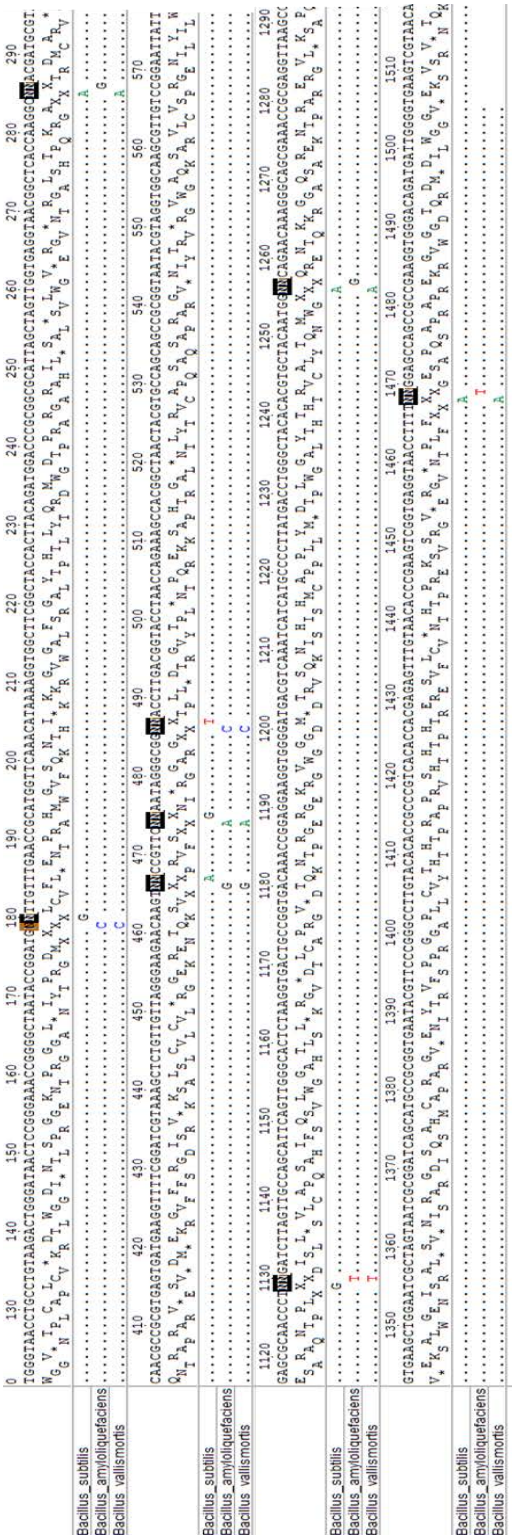

Figure 21. Alignement of *B. vallismortis, B. subtilis* and *B. amyloquefaciens* 16S partial sequences from NCBI database

1.1.2.2 Precision and reproducibility of Vitek-MS

Precision of the method was assessed by performing ten repetitions of the identification of two reference microorganisms: *E. coli* ATCC 8739 and *M. luteus* ATCC10240.

Results obtained from all replicates coincided with its corresponding reference microorganism and the percentage of confidence obtained for each one was 99%.

In order to assess reproducibility of the method, a different analyst repeated this assay. Differences between both assays weren't detected (data not shown).

1.1.3 Validation of PCR-Sequencing method

1.1.3.1 Accuracy and reproducibility of PCR-Sequencing system

Accuracy for the molecular identification system was assessed by performing the identification of reference microorganisms. In contrast with the validation of Vitek-MS, internal strains were also used: Sep_SJD, Bcep_SJD, BZC3, BZC11, BZC22 and BZD41. Results obtained during accuracy assessment are summarized in table 8.

Regarding results from table 8, identifications obtained for each isolate coincide with their reference microorganism. Percentage of identity for each sample is shown and it is higher than ninety.

This same assay was repeated in a different day to assess reproducibility. Differences between days aren't observed.

Table 8. Assessment of accuracy in PCR-Sequencing method

Strain of reference[a]	ID result[b]	% Identity[c]
Bacillus subtilis ATCC®6633	+	99%
Escherichia coli ATCC®8739	+	99%
Enterococcus hirae ATCC®10541	+	99%
Micrococcus luteus ATCC®10240	+	100%
Staphylococcus aureus ATCC®6538	+	99%
Staphylococcus epidermidis ATCC®12228	+	99%
Staphylococcus epidermidis (Sep_SJD)	+	99%
Staphylococcus haemolyticus (BZC3)	+	99%
Staphylococcus hominis (BZC11)	+	99%
Kytococcus schroeteri (BZC22)	+	99%
Kocuria rhizophila ATCC®9341	+	100%
Kocuria palustris (BZD41)	+	100%
Burkholderia cepacia (Bcep_SJD)	+	99%
Pseudomonas aeruginosa ATCC®9027	+	100%

[a]Two replicates were assayed for each strain.
[b]+, the identification is coincident with the strain of reference.
[c]The criteria for accepting the results was a percentage of confidence higher than 90%.

An example of a consensus sequence which was the result of aligning 4 sequences corresponding to primers 27f, 786f, 786Rv and 1492Rv is shown in figure 22. The sequence shown corresponds to the isolate *B. cepacia* 001_SJD_BURK.CEPAC.

>B.cepacia_consensus

NCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGG
CGAGTGGCGAACGGGTGAGTAATACATCGGAACATGTCCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATA
CCGCATACGATCTACGGATGAAAGCGGGGGACCTTCGGGCCTCGCGCTATAGGGTTGGCCGATGGCTGATTAGCTAG
TTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGC
GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCTTGGCTCTAATACAGTCGGGGGATG
ACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGG
AATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGCTAAGACCGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA
TTGGTGACTGGCAGGCTAGAGTATGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGG
AGGAATACCGATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACG
CGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGT
GGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGTCGGAATCCTGCTGAGAGGTGG
GAGTGCTCGAAAGAGAACCGGCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGT
TGCCAACCCGCGAGGGGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGAGTGCATGAA
GCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACAC
CATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCTAACCGCAAGGAGGACGGTCACCACGGTAGGATTCATGACTGGG
GTGAAGTCGTC

Figure 22. Example of consensus sequence with highlighted primer annealing regions: green, blue and yellow for 27f, 786f and 1492Rv respectively

1.1.3.2 Precision and reproducibility of PCR-Sequencing system

Precision was assessed following the same approach described in the validation of Vitek-MS, using *E. coli* ATCC 8739 and *M. luteus* ATCC 10240 and ten repetitions of each isolate. Results obtained for the ten repetitions of each species coincided with their reference microorganism. Percentage of identity was higher than ninety for every isolate.

To assess reproducibility, all strains were identified in two different days and no differences were observed between them.

1.2 Comparison of Vitek-MS and PCR-Sequencing methods

1.2.1 Identification by DNA sequencing method of an in-house microbial collection

Reig Jofre had an old collection of microbial strains isolated from water analysis, environmental monitoring and product testing that were maintained in glycerol at -20°C. This in-house collection had been identified by MALDI-TOF methodology, specifically

BRUKER technology. Taking advantage of this, it was decided to study all this collection by PCR-Sequencing method.

First of all, each strain was recovered from glycerol, was recultured and transferred to Protect® system, which allows long storage of microorganisms at -80°C. In addition, microorganisms recultured were identified by PCR-Sequencing, in order to check if the original identification was maintained.

Glycerol preserved bacterial samples were recultured in TSA media and incubated for at least 24h between 32.5 ± 2.5°C until enough cellular mass was obtained. Bacterial pure cultures were obtained, and morphology of each strain was determined by Gram staining.

Fungal isolates were recultured in Saboureaud Dextrose Agar 4% and incubated at least 2 days at 22.5° ± 2.5C until enough cellular mass was obtained. Yeast isolates were stained with methylene blue while filamentous fungi were observed fresh without the need for staining and their morphology was taken as a preliminary identification.

Data corresponding to microscope images of bacterial Gram-staining and fresh cuts of fungi are gathered in Annex I.

Bacterial and fungal strains samples were identified by *16S* and *18S* sequencing respectively. Results obtained were compared to those from MALDI-TOF, although samples used for sequencing were timely different from the originals used for MALDI-TOF (Table 9).

As it appears in table 9, the majority of isolates previously identified by MALDI-TOF method correspond to Gram positive Bacteria: cocci, bacillus and rods. Some Gram negative rods have also been detected and stored. In a lower extent filamentous fungi were found, mainly belonging to *Aspergillus* genus and yeast found corresponded to *Rhodotorula mucilaginosa*.

Table 9. Comparison of MALDI-TOF and PCR-Sequencing methods in identification of in-house microbial strains

Strain [a]	*MALDI-TOF* [b]	Match Score[c]	Sequencing [d]	% ID[e]
BACID1	*Paenibacillus glucanolyticus*	*2,393*	*+*	99
BACID4	*Bacillus circulans*	*2,080*	*+*	99
BACID5	*Bacillus simplex*	*2,102*	*+*	99
BACID6	*Bacillus simplex*	*1,920*	*+*	99
BACID10	*Bacillus aryabhattai*	*2,382*	*+*	99
BACID20	*Bacillus jeotgali*	*1,924*	*+*	99
BACID28	*Bacillus sonorensis*	*2,468*	*+*	99
BACID34	*Bacillus sonorensis*	*1,999*	*Bacillus licheniformis*	99
BACID32	*Bacillus infantis*	*2,272*	*Dermacoccus barathri*	99
BACID3	*Corynebacterium xerosis*	*1,886*	*Staphylococcus saprophyticus*	99
BACID16	*Corynebacterium aurimucosum*	*2,296*	*Bacillus licheniformis*	99
BACID24	*Corynebacterium mucifaciens*	*2,037*	*Corynebacterium ureicelerivorans*	99
BACID2	*Staphylococcus cohnii*	*2,244*	*+*	100
BACID11	*Staphylococcus epidermidis*	*2,201*	*+*	99
BACID12	*Staphylococcus haemolyticus*	*1,751*	*+*	99
BACID13	*Staphylococcus saprophyticus*	*1,894*	*+*	99
BACID51	*Staphylococcus epidermidis*	*1,835*	*Staphylococcus haemolyticus*	99
BACID52	*Staphylococcus sleifi*	*1,921*	*Staphylococcus pettenkoferi*	99
BACID14	*Micrococcus luteus*	*2,426*	*+*	99
BACID17	*Micrococcus cohnii*	*2,248*	*+*	100
BACID7	*Pseudomonas perfectomarina*	*1,770*	*Pseudomonas stutzeri*	99
BACID36	*Stenotrophomonas maltophilia*	*2,338*	*+*	99
BACID23	*Pantoea brenneri*	*2,435*	*Pantoea allii*	98
BACID50	*Burkholderia cepacia*	*2,562*	*Burkholderia cepacia*	99
BACID53	*Ralstonia picketii*	*2,206*	*Moraxella osloensis*	99
FGID1	*Aspergillus flavus flavus/ oryzae oryzae*	*2,196*	*Aspergillus oryzae*	96
FGID10	*Aspergillus quadrilineatus/parvathecius*	*2,395*	*Aspergillus oryzae / terreus*	98
FGID17	*Aspergillus repens/ ruber*	*2,229*	*Aspergillus terreus*	98
FGID22	*Aspergillus ochraceus*	*2,413*	*+*	99
FGID7	*Penicillium flavigenum/chrysogenum*	*2,165*	*Penicillium chrysogenum*	99
FGID29	*Rhodotorula mucilaginosa*	*1,994*	*+*	99

a) Internal code given to each strain.
b) Results obtained with MALDI-TOF
c) Match score = percentage of confidence of the identification result. Acceptable match score is higher than 1.75
d) Results obtained with PCR-Sequencing; + indicates that result coincides with MALDI-TOF method.
e) Percentage of confidence of the identification result. Acceptable percentage is higher than 90.

Both identification systems agree in all genus determination except for four isolates: BACID3, BACID16, BACID32 and BACID53. Figure 23 shows the Gram-staining of these last four isolates, which is coherent with the results obtained by PCR-Sequencing. At species level, several isolates show differences between MALDI-TOF and PCR-Sequencing: BACID24, BACID34, BACID51, BACID52, BACID7, FGID10 and FGID17. The confidence level shown in the table for each method indicates that the identification achieved was correct but a

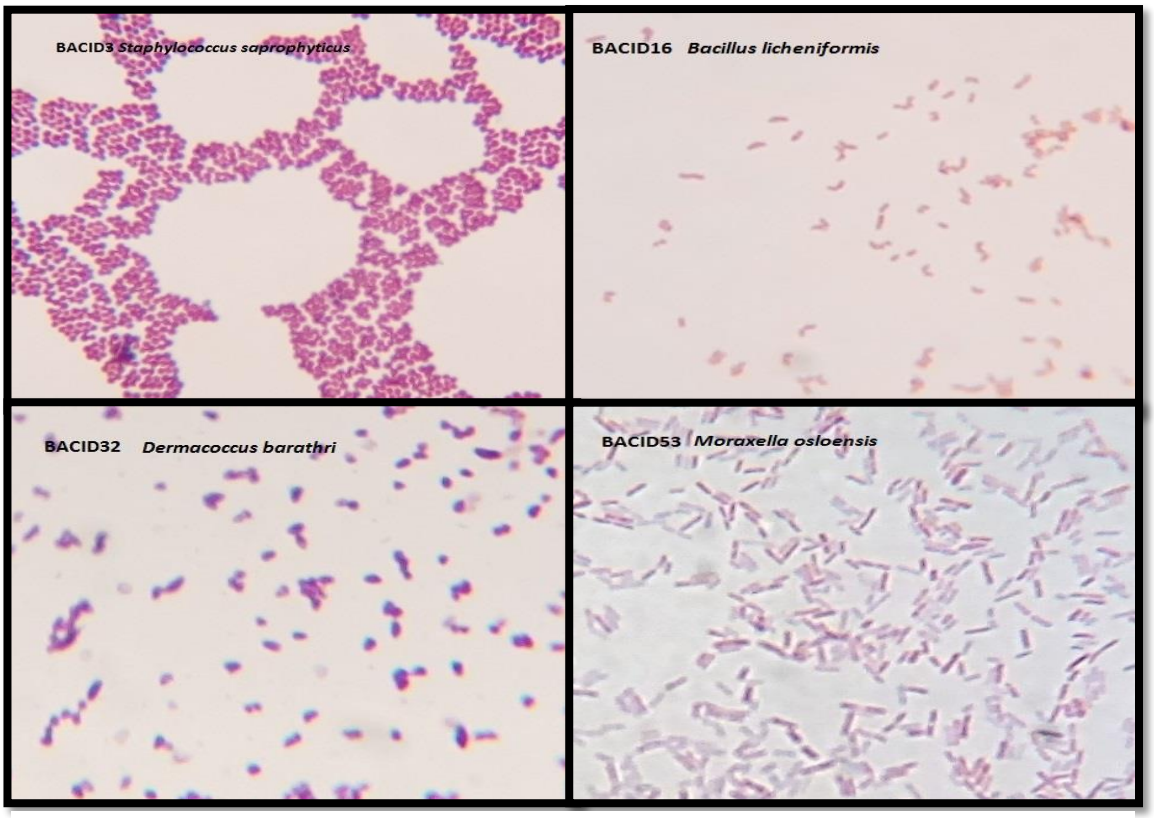

Figure 23. Gram-staining of strains whose genus identification was different between MALDI-TOF and PCR-Sequencing methods

1.2.2 Identification of microbial strains isolated from cleanrooms by Vitek-MS and PCR-Sequencing methods

Strains recovered from air monitoring of cleanrooms were identified in parallel by Vitek-MS and PCR-Sequencing methods. Cleanrooms belonged to C and D classes, which are the less restrictive in particle content. Strains were isolated from culture plates obtained by active air sampling. From a total of 10 plates from every room, different strains were picked regarding colony morphology and identified by both methods.

In table 10, it can be seen that Vitek-MS failed to give an identification from seven of the strains: BZC5, BZC21, BZC22, BZC8, BZC4, BZD33, BZD45 and BZD29. In contrast, PCR-Sequencing method succeed in identifying all strains. Identification results obtained by PCR-Sequencing for those strains were *S. haemolyticus*, *S.capitis*, *Kytococcus schroeteri*, *Globicatella sanguini*, *S. hominis*, *Brachybacterium spp* and *Bacillus circulans* respectively. Recovery of Vitek-MS in comparison with PCR-Sequencing, which has a 100% identification success of these strains, WAs 85%.

At genus level, results from both methods coincide except for strain BZD37, identified as *Staphylococcus epidermidis* by Vitek-MS methods and as *Kocuria rhizophila* by PCR-Sequencing methods. At the species level, there are slight differences; for example, BZC13 and BZC16 were identified as *M.luteus* by Vitek-MS while PCR-Sequencing identified them as *M. yunanensis*. In addition, BZC5 was identified as *S. caprae* by Vitek-MS while *S. capitis* by PCR-Sequencing. For the strains which had an identification result by both methods, coincidence of results was higher than 90%.

Table 10. Identification of environmental isolates by Vitek-MS and PCR-Sequencing

CLASS	ISOLATE	VITEK-MS[a]	PCR-SEQUENCING[b]
C	BZC3	*Staphylococcus haemolyticus*	+
	BZC5	*Staphylococcus caprae*	*Staphylococcus capitis*
	BZC7	-	*Staphylococcus haemolyticus*
	BZC10	*Staphylococcus haemolyticus*	+
	BZC11	*Staphylococcus hominis*	+
	BZC14	*Staphylococcus haemolyticus*	+
	BZC19	*Staphylococcus haemolyticus*	+
	BZC20	*Staphylococcus caprae*	*Staphylococcus capitis/caprae*
	BZC21	-	*Staphylococcus capitis*
	BZC24	*Staphylococcus haemolyticus*	+
	BZC26	*Staphylococcus haemolyticus*	+
	BZC27	*Staphylococcus haemolyticus*	+
	BZC1	*Micrococcus luteus*	+
	BZC2	*Micrococcus luteus*	+
	BZC6	*Micrococcus luteus*	+
	BZC9	*Micrococcus luteus*	+
	BZC12	*Micrococcus luteus*	+
	BZC13	*Micrococcus luteus*	*Micrococcus yunnanensis*
	BZC15	*Micrococcus luteus*	+
	BZC16	*Micrococcus luteus*	*Micrococcus yunnanensis*
	BZC17	*Micrococcus luteus*	+
	BZC23	*Micrococcus luteus*	+
	BZC25	*Micrococcus luteus*	*Micrococcus spp.*
	BZC28	*Micrococcus lylae*	+
	BZC8	-	*Kytococcus schroeteri*
	BZC22	*Kytococcus schroeteri*	+
	BZC4	-	*Globicatella sanguini/suldifaciens*
D	BZD32	*Staphylococcus hominis*	+
	BZD33	-	*Staphylococcus hominis*
	BZD34	*Staphylococcus epidermidis*	+
	BZD43	*Staphylococcus capitis*	*Staphylococcus capitis*
	BZD44	*Staphylococcus hominis*	+
	BZD47	*Staphylococcus hominis*	+
	BZD30	*Micrococcus luteus*	+
	BZD35	*Micrococcus luteus*	+
	BZD39	*Micrococcus luteus*	+
	BZD40	*Micrococcus luteus*	+
	BZD42	*Micrococcus luteus*	+
	BZD46	*Micrococcus luteus*	+
	BZD31	*Kocuria rhizophila*	+
	BZD36	*Kocuria rhizophila*	+
	BZD37	*Staphylococcus epidermidis*	*Kocuria rhizophila*
	BZD38	*Kocuria rhizophila*	+
	BZD41	*Kocuria palustris*	+
	BZD45	-	*Brachybacterium spp*
	BZD29	-	*Bacillus circulans*

[a]– indicates failure in identification

[b]+ indicates that PCR-Sequencing result coincided with that of PCR-Sequencing

Figure 24 shows a pie chart with the distribution by genus of the environmental isolates identified by PCR-Sequencing. Legend from figure 24 shows that all 46 isolates belong to 7 different genus. Genus *Staphylococcus* and *Micrococcus* are the most representative ones occupying a 39% of the strains each one, which means 80% of the strains belong to these two genus. Genus *Kocuria* and *Kytococcus* are represented by more than one strain in this set of isolates.

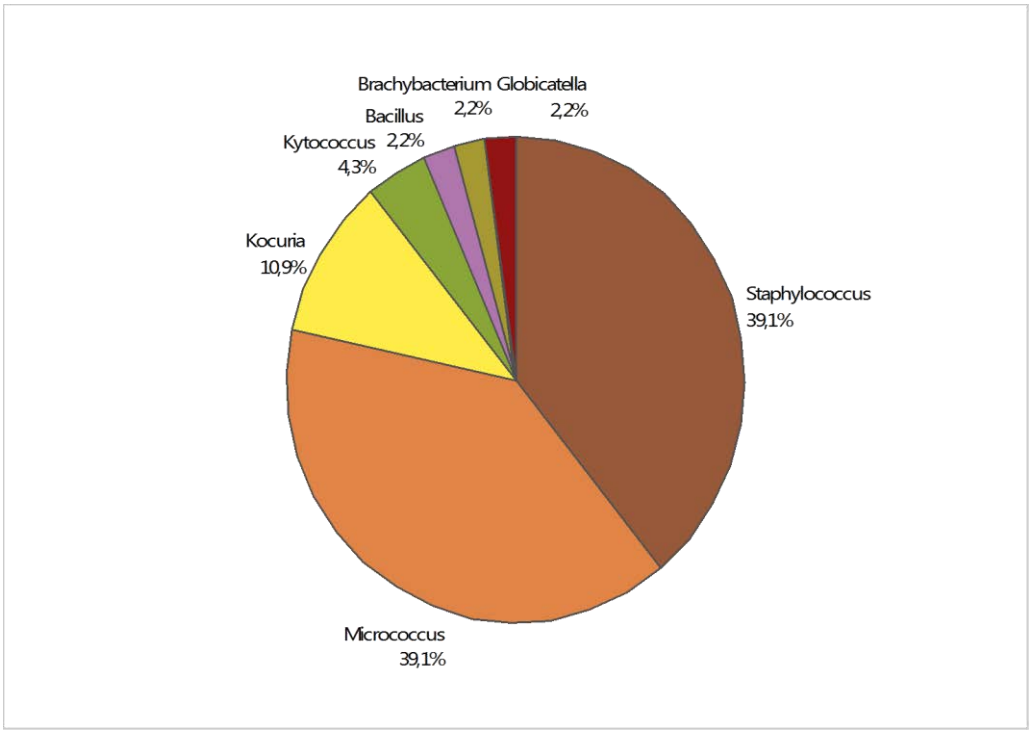

Figure 24. Genus distribution of the environmental isolates by PCR-Sequencing

1.3 Implementation of Vitek-MS and PCR-Sequencing methods

1.3.1 Microbial identification program

The microbial identification program designed after validation of Vitek-MS and PCR-Sequencing is shown in figure 25. Microbial isolates recovered from purified water (PW) and water for injection (WFI), environmental monitoring (cleanroom air, personnel garments, surfaces) and non-sterile products, will be identified by Vitek-MS. Besides, microbial isolates recovered from sterility tests, media fills and contamination

investigation, will be directly identified by PCR-Sequencing. In addition, microbial isolates which Vitek has failed to identify, will be also identified by PCR-Sequencing.

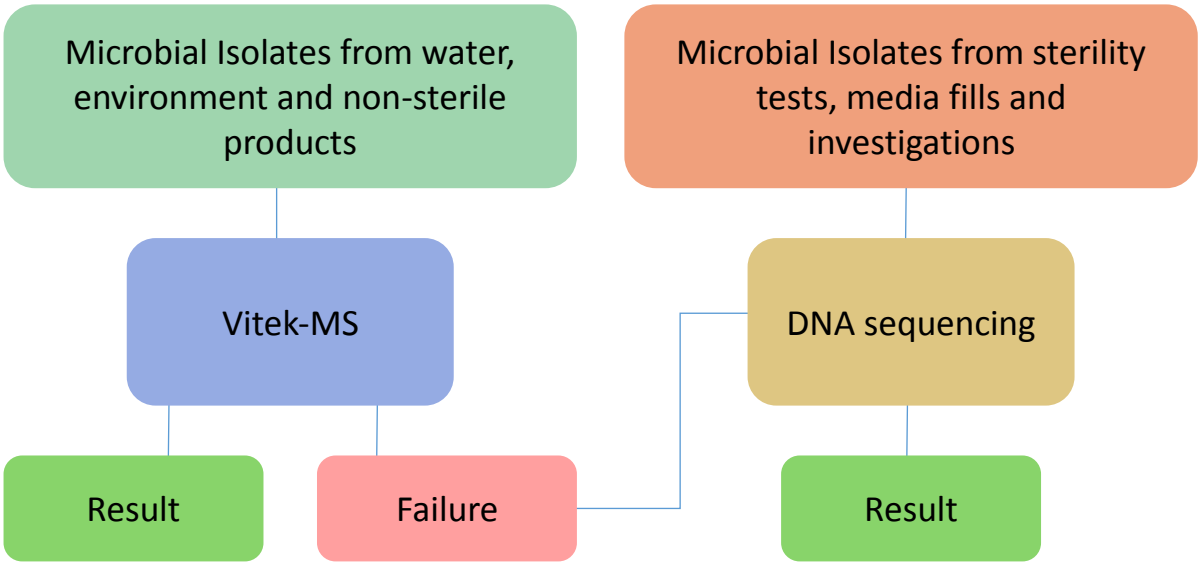

Figure 25. Schematic representation of the microbial identification program

1.3.2 Implementation of Vitek-MS

Vitek-MS was validated and used as the microbial identification system in the routine of the microbiology control laboratory as it is shown in figure 25. A total of 1868 isolates from all the different microbial monitoring points of the manufacturing process and the different products and materials were obtained in a time period of 14 months. From those, 1625 (87%) were identified by Vitek-MS. The remaining 13% failed by this method and, consequently, they were identified by PCR-Sequencing.

Figure 26 shows the distribution of microbial isolates found during 14 months of microbial monitoring, which have been identified by Vitek-MS. Bacteria have been classified according to Gram staining as well as morphology while fungi have been classified between molds and yeasts.

Regarding distribution observed in figure 26, the majority of microorganisms found in the plant correspond to Gram positive cocci (42.8%), such as *Staphylococci* or *Micrococci*. The

secondly most frequent microorganisms are Gram negative rods (25.8%) followed by Gram-positive spore-forming rods (9.9%). The remaining groups have a more similar frequency, being the least common found in the manufacturing plant. From the total of microbial isolates, fungi represent around a 6% of the isolates, being yeasts more abundant than molds.

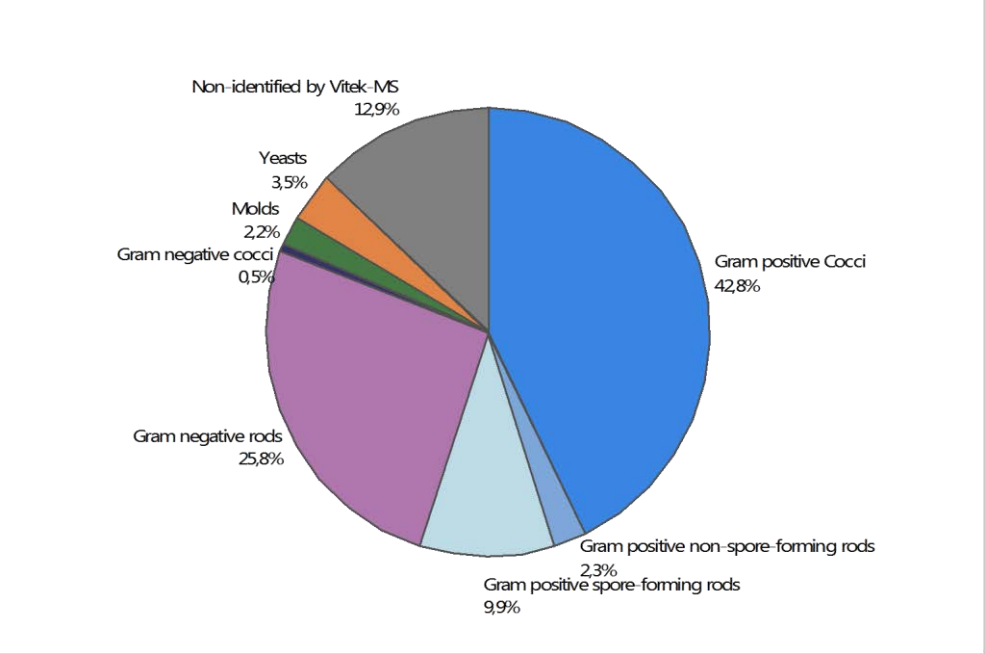

Figure 26. Classification of isolates identified by Vitek-MS

Table 11 describes the distribution of the isolates according to their origin. On the one hand, gram-negative bacillus represent were predominant in WFI and PW, 76% and 86% respectively. On the other hand, gram-positive cocci were the most abundant in cleanroom monitoring, including air, surfaces and personnel. There were slight differences between WFI and PW in the type of microorganisms found in them. For example, 12% of microorganisms identified in WFI were gram-positive cocci while only 3% of this kind were found in PW.

Table 11. Classification of isolates from WFI, PW and cleanroom monitoring of classes A, B, C and D

Classification	WFI	PW	Class A	Class B, C and D
Gram-negative Bacillus	76%	86%	3%	10%
Gram-positive spore-forming bacillus	5%	4%	8%	5%
Gran-positive non-spore forming bacillus	0%	3%	6%	9%
Gram-positive cocci	12%	3%	78%	70%
Gram-negative cocci	3%	3%	0%	1%
Yeast	3%	2%	1%	4%
Molds	0%	1%	4%	2%

Figure 27 shows the number of isolates corresponding to each genus. The last column, named as "others", corresponds to the 10% of the isolates that belong to less representative genus (less than 1%). Some genus included in this group are *Enterobacter*, *Penicillium*, *Moraxella*, *Escherichia* or *Klebsiella*.

The most frequent genus found was *Staphylococcus*, which enclosed 31.0% of the isolates. Following *Staphylococci*, 13% of the isolates found belonged to the genus *Micrococcus* while 10% belonged to *Bacillus*. The following genus were more equally distributed, with percentages between 1 and 4%; among them *Ralstonia*, *Kocuria*, *Pseudomonas* or *Burkholderia* were found. The most abundant fungal genus were *Candida* and *Aspergillus* with 3 and 4% frequency respectively.

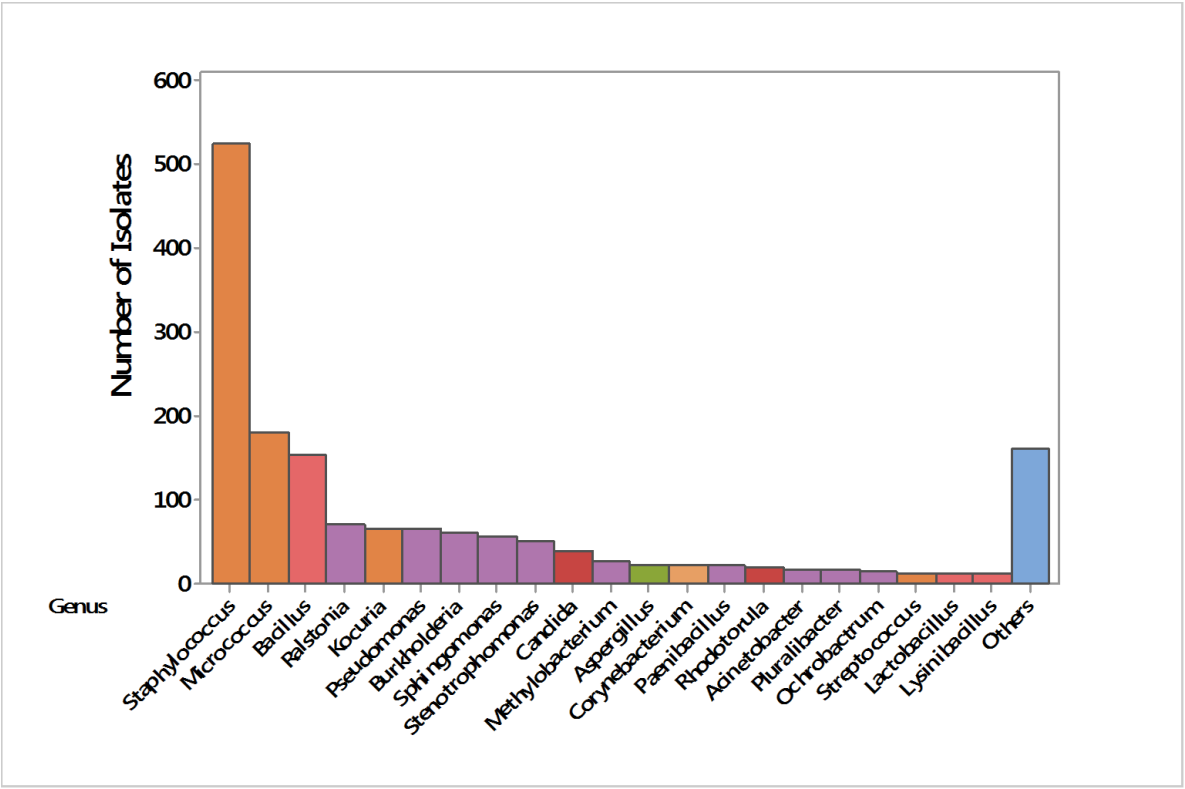

Figure 27. Distribution of isolates identified by Vitek-MS classified by genus

1.3.3 Implementation of PCR-Sequencing

According to the microbial identification program shown in figure 25, PCR-Sequencing method was implemented as a backup for Vitek-MS and identification of isolates from specific origin. Vitek-MS failed to identify 13% of the isolates in the time period from Apr-2017 to June-2018.

Table 12 summarizes the species that have been identified by PCR-Sequencing method and cannot be found among the results generated by Vitek-MS. Results are classified according to Gram-staining and morphology.

At the genus level, the majority of genus shown in table 12 were found within the Vitek-MS database. However, this method failed in their identification in given isolates.

Table 12. Identification results obtained by PCR-Sequencing that weren't identified by Vitek-MS method

	Microorganisms identified by Sequencing
Gram-positive cocci	*Brachybacterium conglomeratum*
	Brachybacterium sp.
	Micrococcus cohnii
	Nesterenkonia lacusekhoensis
	Staphylococcus devriesei
Gram-positive non-spore-forming rods	*Corynebacterium auriscanis*
	Corynebacterium efficiens
	Leifsonia lichenia/shinshuensis
Gram-positive spore-forming rods	*Bacillus beijingensis*
	Bacillus flexus
	Bacillus niacini
	Oceanobacillus caeni
	Paenibacillus camelliae
	Paenibacillus chitinolyticus
	Paenibacillus humicus
	Paenibacillus turicensis
Gram-negative rods	*Afipia genomospecies*
	Azospirillum sp.
	Dyella japonica
	Dyella spp
	Massilia aurea
	Methylobacterium dextranolyticum/ flavescens
	Methylobacterium persicinum
	Methylobacterium populi
	Paracoccus acridae
	Paracoccus aestuarii / carotinifaciens / marcusii
	Phyllobacterium myrsinacearum
	Sphingomonas aquatilis / melonis
	Variovorax paradoxus
	Xenophilus aerolatus
Molds	*Acremonium brunnescens*
	Alternaria botrytis / papavericola
	Cladosporium halotolerans
	Scopulariopsis brevicaulis
	Trichoderma atroviride

2 Implementation of a laser-induced fluorescence system for the environmental monitoring of cleanrooms

Cleanroom air in an aseptic manufacturing facility needs to be monitored for non-viable particles as well as viable particles[6]. Viable particles have always been detected as colony forming units (CFU) as the methods available are agar-based. Sampling of air for microbial monitoring is performed by passive or active impaction on agar plates.

One of the objectives of this project was the implementation of a nearly real-time laser-induced fluorescence system in the monitoring of viable particles in cleanroom air of the aseptic processing facility. The equipment chosen was Biolaz®, which has been qualified for its use in routine and alert and action limits have been established for its operation in the environmental monitoring program.

2.1 Validation of the laser-induced fluorescence system and qualification for the intended use

Following the guidelines for implementation of new methods in the pharmaceutical processing facility, the system must be validated according to Pharmacopoeia. Validation of alternative methods in microbiology requires specific guidance, which is gathered in chapters “Alternative methods for control of microbiological quality” from EP and chapter “Validation of alternative microbiological methods” from USP[82,83].

The validation approach for this system consists of the qualification of the equipment for its operation in the facility. Qualification consists of 3 components: installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ). These three components are defined as following:

- IQ: establishing by objective evidence that all key aspects of the process equipment and ancillary system installation adhere to the manufacturer’s approved specification and that the recommendations of the supplier of the equipment are suitably considered[84].
- OQ: establishing by objective evidence process control limits and action levels which result in product that meet all predetermined requirements[84].

- PQ: establishing by objective evidence that the process, under anticipated conditions, consistently produces a product which meets all predetermined requirements [84].

As a global, IQ, OQ and PQ are intended to ensure that the equipment accomplishes with the demands of the company and operates well in base to its own characteristics and the specifications indicated by GMP guidance.

2.1.1 Installation and Operational qualification of the laser-induced fluorescence system

Usually, the IQ/OQ of equipment corresponds to the manufacturer's duty. The IQ/OQ was performed and successfully completed prior to this study by PMS technical support in Spain (affiliated with IESMAT), and all aspects evaluated were in compliance.

Parameters that are evaluated during IQ are, among others, energy supply, space availability or room temperature requirements for the optimal functioning of the equipment. During the OQ it is tested that software and equipment connect well once located in its final position and that the system operates well according to manufacturer's specifications.

2.1.2 Performance qualification of the laser-induced fluorescence system part I

The first part corresponding to performance qualification (PQ1) corresponds to the validation of the system performed by the manufacturer. Validation is performed in a general basis according to the requirements stated by Pharmacopoeia for a quantitative system.

Parameters assessed during validation were Accuracy, Precision, Specificity, Detection Limit, Quantification Limit, Linearity, Operational Range, Robustness, Repeatability and Ruggedness. The last two parameters are only required by USP <1223>[82].

The system was validated using different microorganisms: Gram-positive cocci (*Staphylococcus epidermidis)* and Gram-positive rods (*Bacillus subtilis*, *globigii* and

thuringensis), in their vegetative and spore forms. In addition, *Aspergillus* and yeast were also used.

The alternative system Biolaz was assessed versus the traditional method of agar-based active-air sampling method Air-trace. Preparations of the above-mentioned microorganisms were introduced into a particle generation system that dispenses a known concentration of particles in the form of an aerosol. The traditional and alternative methods are located in parallel in such a way that they receive the same aerosol content. Microbial recovery was quantified for each system and they were compared, concluding that the alternative method Biolaz complied with all parameters evaluated and is suitable for quantification of microorganisms in air.

Results obtained in this validation were generated by the manufacturer Particle Measurements Systems (PMS) and they were gathered in a validation report[53].

2.1.3 Performance qualification of the laser-induced fluorescence system part II

Given that the system has been validated by the manufacturer according to Pharmacopoeia, it needs to be qualified to its intended use in its final location now.

This part is named PQ2 and must be performed by the user in the facility to be implemented in. It consists of the data generation on site, demonstrating that it is suitable for its use in environmental monitoring of cleanroom air, and the setting of alert and action limits.

The alternative method was compared to the traditional agar-based active air sampling method Sampl'air.

To perform PQ2, ten measures of different air volumes were taken simultaneously with the active air sampling method and laser-induced fluorescence system in Classes A, B, C and D of cleanroom facilities, for both "at rest" and "in operation" conditions. In Class A, ten measures were taken in operation but only 5 measures were taken at rest because of the tight manufacturing schedule of the room, as it was fully functioning at the time.

Figure 28 shows a schematic representation of the sampling strategy followed during validation, which is the same strategy that will be used in the routine manufacturing process. Active-air sampling is performed after material preparation and before starting the filling process. Passive air sampling plates are located in the dosification area and changed every 4 hours if the dosification period is higher than this time. Biolaz is sampling continuously in parallel to the previous two methods and stops only if the power is cut.

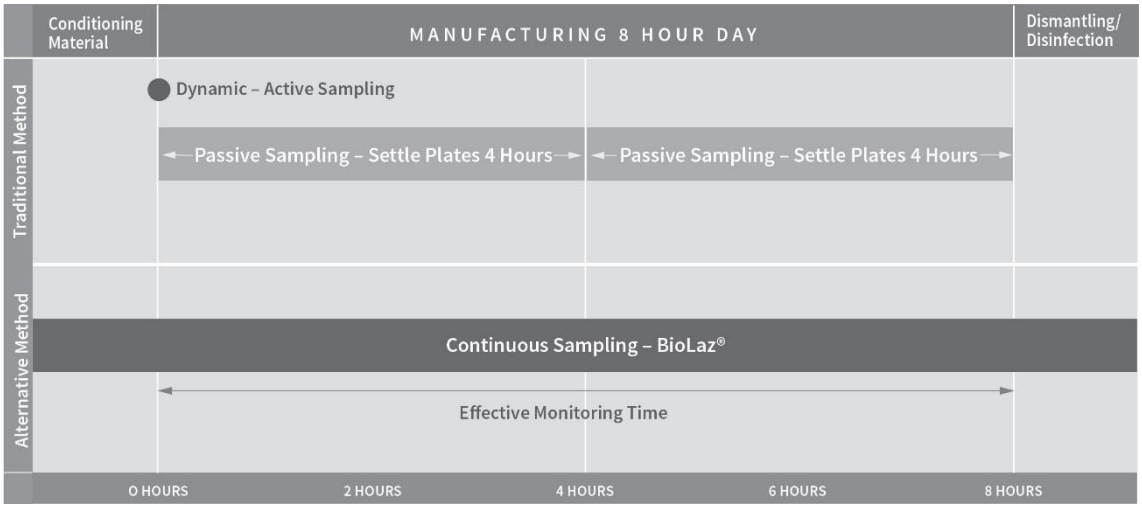

Figure 28. Schematic representation of sampling strategy for air monitoring in routine processes

The mean values of Biocounts and CFUs are summarized in Table 13, along with standard deviation for every classified area, for both the alternative and traditional method. It was expected that Biocounts and CFU in operation conditions were higher than at rest conditions. In class C, average Biocounts are higher at rest than in operation conditions, however, regarding the large standard deviations, no real numerical difference between both conditions was observed. The same explanation can be applied to average CFU from classes C and D. CFU values for class D are rarely low taking into account that the highest particle levels are expected in this class. However, it is worth to note that class D in which measures were taken wasn't operating fully.

Table 13. Viable particles detected in cleanrooms by traditional and alternative methods

		Alternative method (Biocounts/m³)		Traditional method (CFU/m³)	
	Condition	**Average**	**SD**	**Average**	**SD**
ClASS A					
	IO	26	26	0	0
	AR	16	9	0	0
CLASS B					
	IO	328	141	2	5
	AR	16	18	0	0
CLASS C					
	IO	5239	3683	33	21
	AR	8613	7760	45	24
CLASS D					
	IO	1904	1407	15	7
	AR	859	425	24	16

In order to compare Biocounts and CFU obtained from every cleanroom and condition, data from both systems were standardized. Value standardization was performed by substracting the average from every room and condition and dividing by the standard deviation. By applying this equation, average of normalized data results 0, so median is used for comparison. Figure 29 shows standardized data plotted with the median and standard deviation of scaled data. Variability of both methods is very high, showing no real differences between conditions in each class.

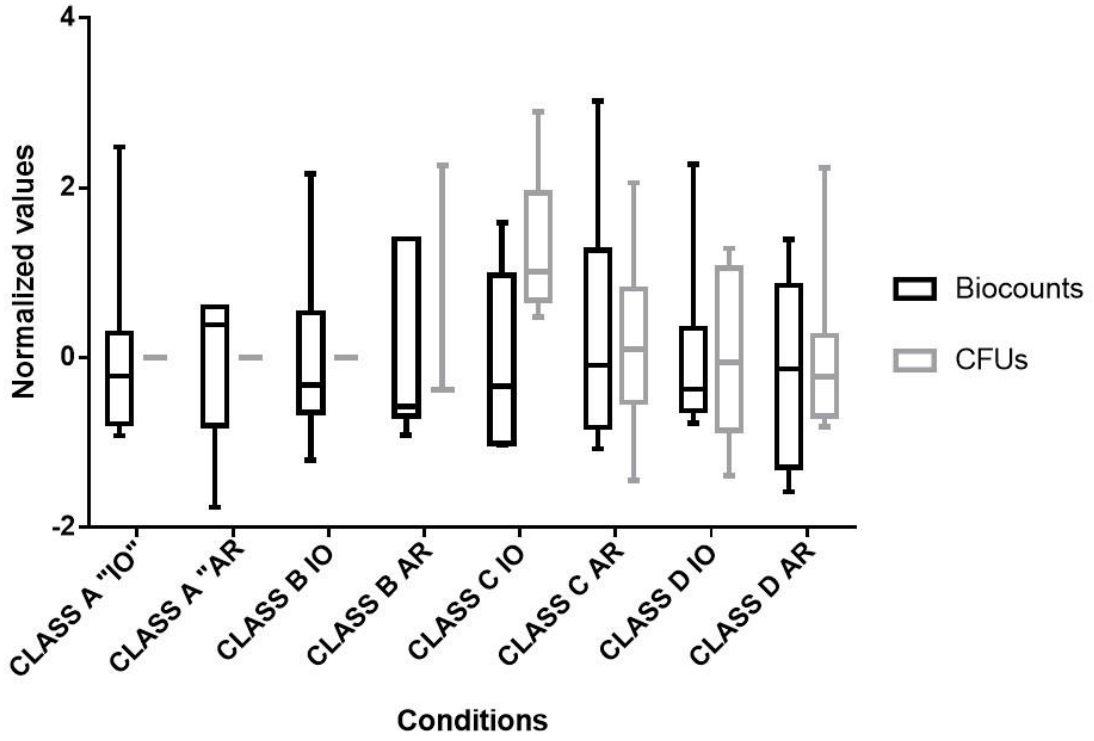

Figure 29. Standardized viable particles in cleanrooms measured by both the traditional and alternative methods

An equivalence test was run in order to compare the alternative method to the traditional one, with a level of significance of 0.95. The results from the equivalence test stated that data from Biolaz and Sampl'air were significantly different, with a P = 0.951. For that reason, the alternative method based on laser-induced fluorescence cannot be declared equivalent to the traditional method of agar-based active air sampling. Raw data showed that mean recovery values of Biocounts were significantly higher than values of CFUs.

The ratio of CFU/Biocount was calculated for every area and they are shown in table 14. To do this, the average of Biocounts was divided by the average of CFU for every area obtaining the number of Biocounts generated for every CFU in every classified cleanroom at rest (AR) and in operation (IO). Class A ratios weren't calculated as the average for both conditions is 0 CFU. The ratio means the number of Biocounts that are theoretically equivalent to 1 CFU for every class and condition. In the table, it can be noted that different ratios are obtained depending on the class and conditions evaluated.

Table 14. Ratio between the number of viable particles obtained by the traditional and alternative methods in cleanrooms

Grade	Ratio traditional/alternative
Class B IO	1:211
Class B AR	1:113
Class C IO	1:159
Class C AR	1:192
Class D IO	1:123
Class D AR	1:34

2.1.3.1 Establishing alert and action limits for the alternative method

After PQ, alert and action limits for class A needed to be set up for routine operation. Alert and action levels are only set for class A because the system was only implemented in this room. They are defined as the following:

-Alert level: an established microbial level giving warning of potential drift from normal operating conditions that triggers appropriate scrutiny and follow-up to address the potential problem. Alert levels are always lower than action levels.

-Action level: a level that, when exceeded, indicates a process has drifted from its normal operating range. Results that exceed action levels are known as excursions. Response to excursions involve a documented investigation and corrective action.

Alert and action levels were calculated in base to internal data, particularly, Biocount data obtained from Class A "in operation". A non-parametric tolerance limit approach was used for statistical calculation of the limits, given that data didn't follow a normal distribution [14]. Taking into account readings obtained during filling operations in class A, there was a drift towards 0 in the values, which is shown in figure 30. When looking at data obtained from filling time only, 0 Biocount readings were frequently observed, with an average of just 26 Biocounts/m^3.

Biocount readings were obtained by the system every 10 seconds. Firstly, Biocounts were added to obtain number of Biocounts/min. However, as the persistence of Biocounts for a short period of time is more likely an indication of contamination, data was evaluated after every 10 minutes, so readings were added in groups of 60 to obtain Biocounts/ 10 min.

Figure 30 shows the distribution of Biocounts/10 min obtained during the filling operations in class A. A total of 345 measures were obtained during this period, with values from 0 to 9 Biocounts every 10 minutes. Tolerance limits approach statistical test was applied to data shown in figure 30. Then, alert level was obtained with confidence level of 95% for 95% of the data while action level was obtained with confidence level of 95% for 99% of the data. Alert and action limits are shown in figure 90, indicating that 95% of data are below 6 Biocounts/10 min and 99% of the data are below 9 Biocounts/10 min.

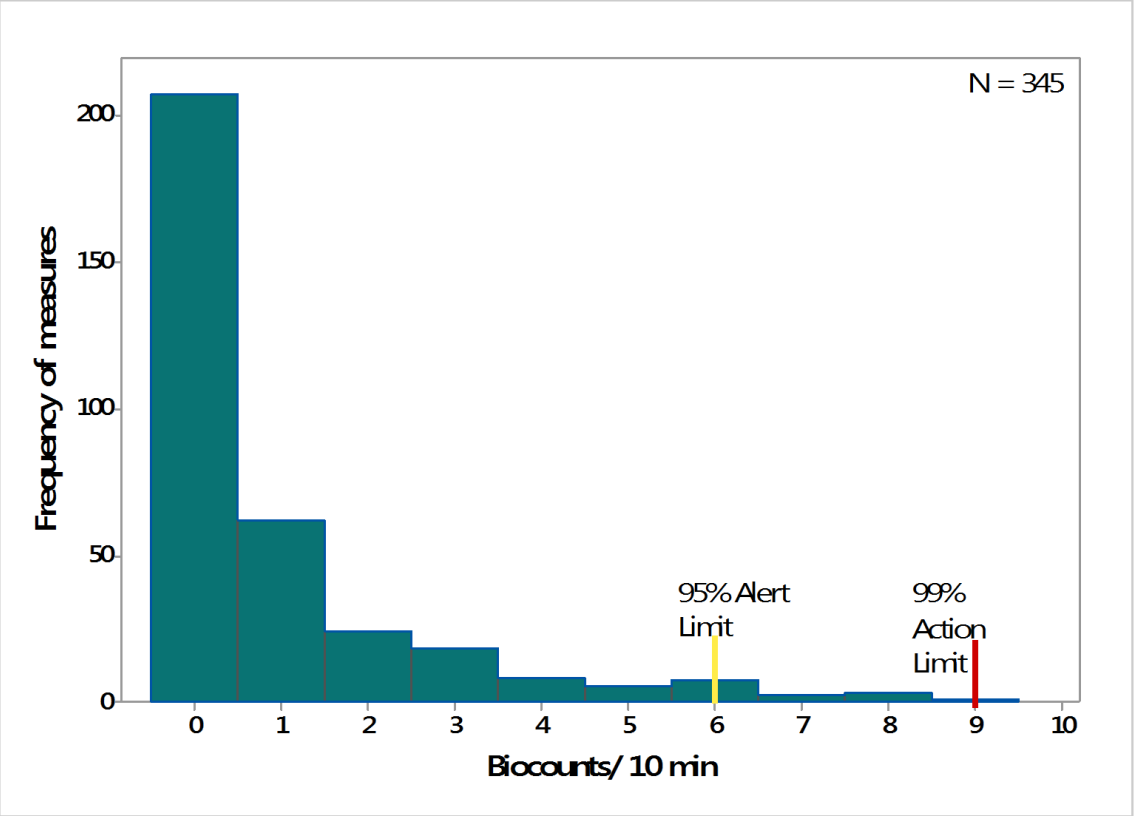

Figure 30. Histogram of Biocounts/10 minutes of class A readings during filling

2.2 Implementation of laser-induced fluorescence system as a method to detect viable particles in an aseptic manufacturing process

Once the system was fully qualified and alert and action limits defined, it was located in its final position for operation during aseptic filling in class A. Biolaz® was located at the most critical point of manufacturing operations, which is aseptic filling. The laser-induced fluorescence system probe collects air that is in contact with vials being filled.

Data from the already qualified laser-induced fluorescence system obtained from June to December 2017 were evaluated. Control charts for every month were performed to obtain Biocount readings every 10 minutes, for 24 hours every day. Note that this means not only taking into account filling time, but also preparation and other activities. Biocounts detected every 10 minutes were added and the avearage for every month was calculated along with standard deviation of the mean. Figure 31 represents the average of Biocounts/10 minutes obtained for every month with the standard deviation.

Regarding figure 31, the average number of Biocounts read every 10 minutes was very similar in June and July, with about 1 Biocount every 10 minutes. Despite the large standard deviation, due to non-normality of data (there is a data drift to 0 Bioocunts/10 minutes while large Biocounts/10 min readings are found sporadically), changes in the average Biocounts/10 minutes are observed between months. There was an increase in the number of Biocounts/10 minutes during the month of September, which reached an average of 5 Biocounts every 10 minutes. From October to December, average Biocounts decreased progressively until there were approximately 2 Biocounts every 10 minutes. No further data was available for evaluation due to the removal of the system for qualification at the end of December. Stabilization of low Biocount levels was expected.

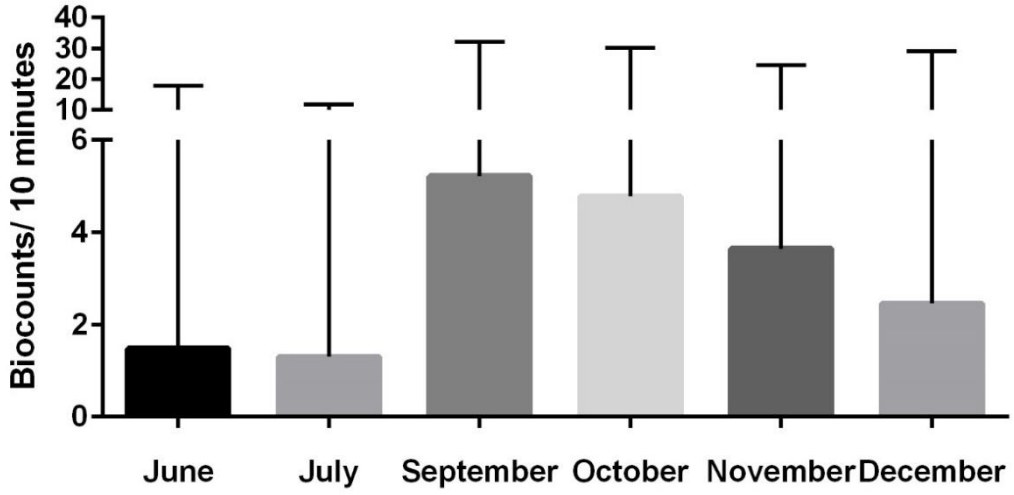

Figure 31. Average of biocounts/10 minutes obtained in an aseptic manufacturing area

To evaluate differences between months in more detail, Biocounts/10 min corresponding only to filling time periods during June and September were evaluated. Figure 32 shows Biocounts detected every 10 minutes during the days when filling operations took place, the horizontal red line labels the action limit. Although the average Biocounts/10 min decrease for both moths when compared to Figure 31, there still was a difference between June and September in the average viable particles detected during filling.

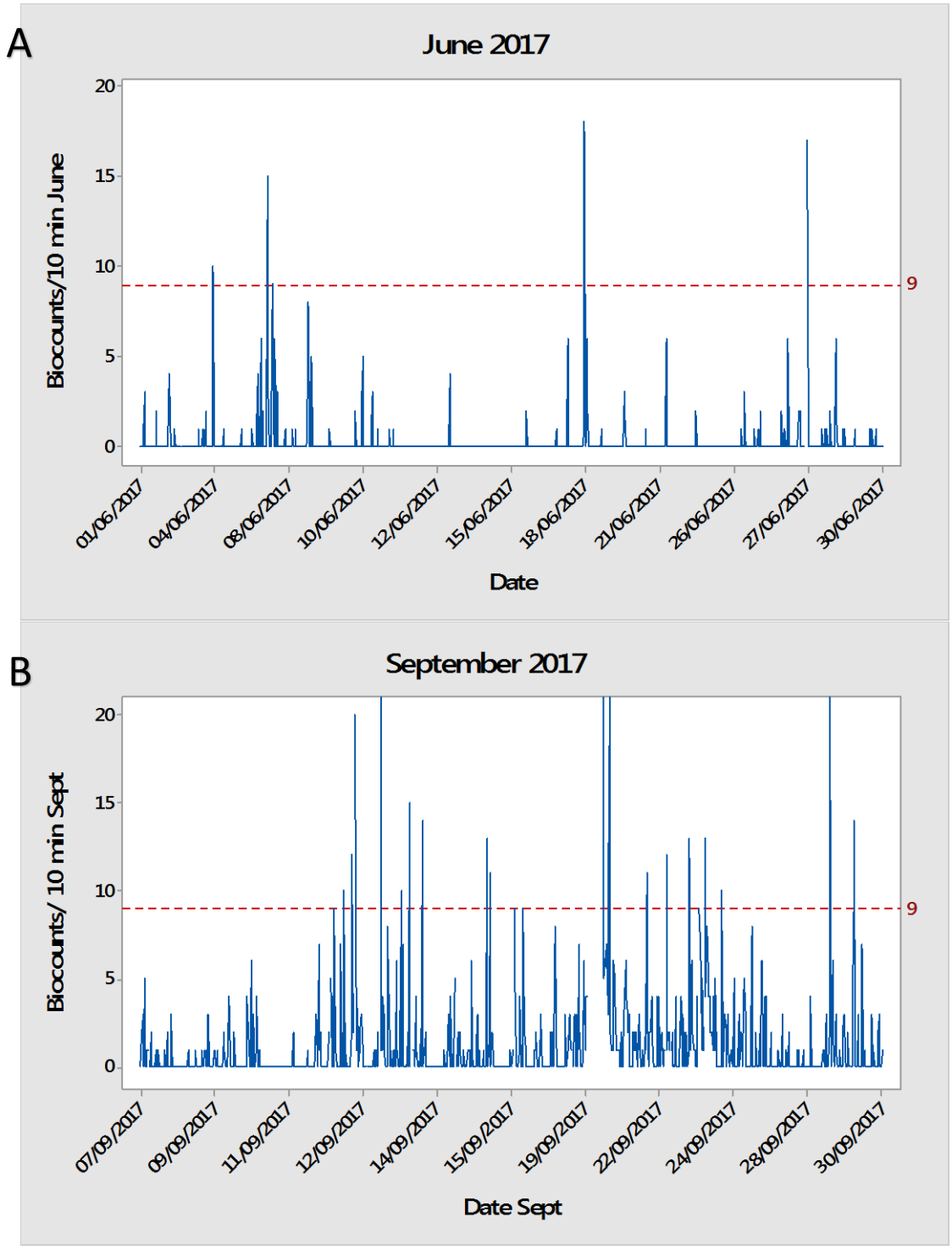

Figure 32. Biocounts/10 minutes detected during filling time periods along June (A) and September (B)

2.2.1 Evaluation of microbial contamination found on plates with data from laser-induced fluorescence system

When action levels are exceeded in microbial environmental monitoring, it means that the process has drifted from the operational range. This fact is named in the pharmaceutical industry with the term excursion. Note that for traditional methods such as settle plates and active-air sampling, action limit is 1 CFU/plate and 1 CFU/m^3 respectively.

During the total time period evaluated, microbial colonies in plate counting were found at different dates: July 16th, September 16th, November 26th and December 12th. The first three corresponded to settle plate control and the last one was found in an active air sampling plate. Biocounts generated by the laser-induced detection system during filling periods on those days were evaluated as part of the investigation of the excursions. As a reminder, alert and action limits set for the laser-induced fluorescence system were 6 and 9 Biocounts/10 minutes respectively.

Biolaz readings from the filling period of the days mentioned above were obtained. Measures were added to obtain Biocounts detected every 10 minutes. Figure 33 shows the filling time period for each day in the X axis and the Biocounts/10 min in the Y axis. Two horizontal lines corresponding to alert (IL) and action limits (SL) are represented in the four graphs. Peaks above action limit of the alternative system were observed at specific moments during filling operations when microbial colonies were detected on plates.

Biolaz data obtained during product filling processes in which any excursion on plates wasn't detected, was compared to the data in figure 33. For example, in data from filling during June 19th or October 9th (data not shown) it doesn't appear any measure above the action limit of laser-induced fluorescence system and any CFU wasn't found on plates either.

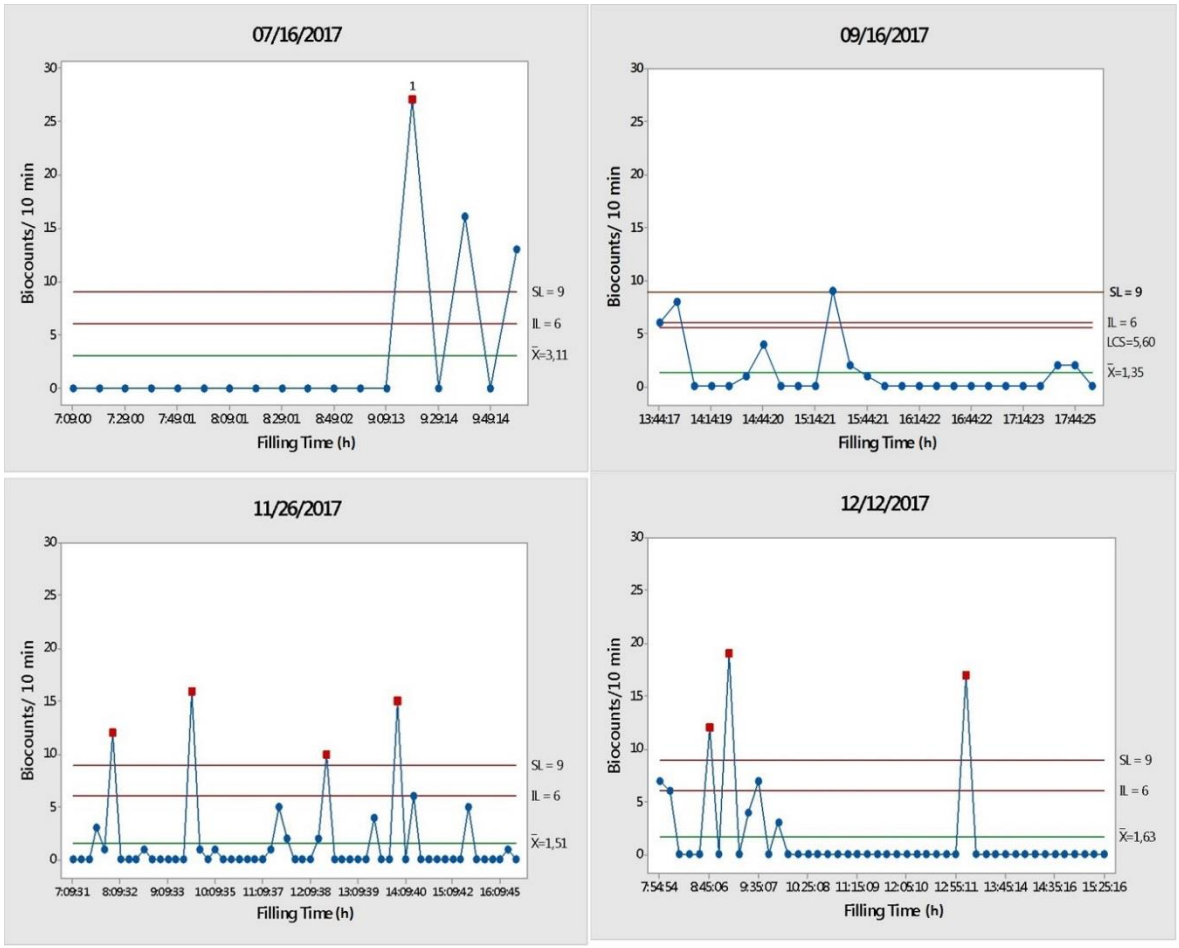

Figure 33. Control charts of biocounts/10 min detected during filling time when excursions on plates were found. Date of the excursion is indicated in every chart

3 Evaluation of a microbial quantification rapid method based on solid-phase cytometry

The third objective of this thesis was to assess a rapid quantitative system for the bioburden analysis of pharmaceutical water and some pharmaceutical products. Hands on the technique were set to evaluate a future implementation of the system in Reig Jofre. From the available methods in the market, a solid-phase cytometry-based system called MuScan® was chosen for trial.

Solid-phase cytometry is a non-growth-based method that detects viable cells using a general stain for viability. In order to test if the system was suitable for implementation in the quality control of the aseptic manufacturing process, several assays were carried out:

1. Firstly, the quantitative capability of the system was evaluated, using two different species of microorganisms and known quantities of inoculum, comparing MuScab method with traditional Milliflex method.
2. Secondly, samples of water for injection (WFI) from the company stream were analyzed in parallel by MuScan and Miliflex method.
3. Finally, bioburden of two pharmaceutical products was assessed in parallel by MuScan and Milliflex method.

All the experiments carried out with MuScan system were performed at Innosieve Diagnostics facilities in the Netherlands.

3.1 Assessment of the quantitative capability of the system: spiking water with known microbial concentration

In order to compare microbial recovery of the solid-phase cytometry system and the Milliflex plate count system, known concentrations of microorganisms were inoculated to sterile water. Bioballs from two different species were used for inoculum: *P. aeruginosa* (NTC12924) and *C. albicans* (NCPF3179).

Results obtained from this experiment are shown in table 15. Concentrations of bioballs used are shown in the table, triplicates from all concentrations were performed. Number of colonies grown on plate was checked every day up to 3 days of incubation. It is

considered that 1 cell counted in MuScan is equivalent to 1 CFU in Milliflex method. The table shows the result for each replicate as well as the average and standard deviation (SD).

Table 15. Comparison of MuScan and Miliflex methods in detection of microorganisms in sterile water

Microorganisms CFU/100 ml	SPC[1] CFU/100 ml			Average	SD	TS[2] CFU/100 ml			Average	SD
Candida albicans										
10	16	8	8	11	5	9	7	6	7	2
50	38	27	38	34	6	39	22	46	36	12
200	145	132	128	135	9	119	150	176	148	29
Pseudomonas aeruginosa										
10	8	12	5	8	4	10	18	15	14	4
50	48	46	51	48	3	43	47	54	48	6
200	124	132	136	131	6	125	132	124	127	4

[1]SPC: Solid-Phase Cytometry
[2]TS: Traditional System based on plate count

Colonies recovered by both systems in every replicate are quite similar. Both systems fail to recover the highest concentration of inoculum. The number of viable cells detected by MuScan is obtained automatically from the system, so it is not necessary for the analyst to perform manual count. The scanner processes thousands of images of the membrane and returns the ones in which fluorescence has been detected.

Figure 34 shows examples of the scan images where fluorescence was detected. The figure consists of one image corresponding to each inoculum concentration, except the blank control. Differences in morphology of both species of microorganisms can be appreciated. Cells belonging to *P. aeruginosa* are rod-shaped and small while cells belonging to *C. albicans* are round and large.

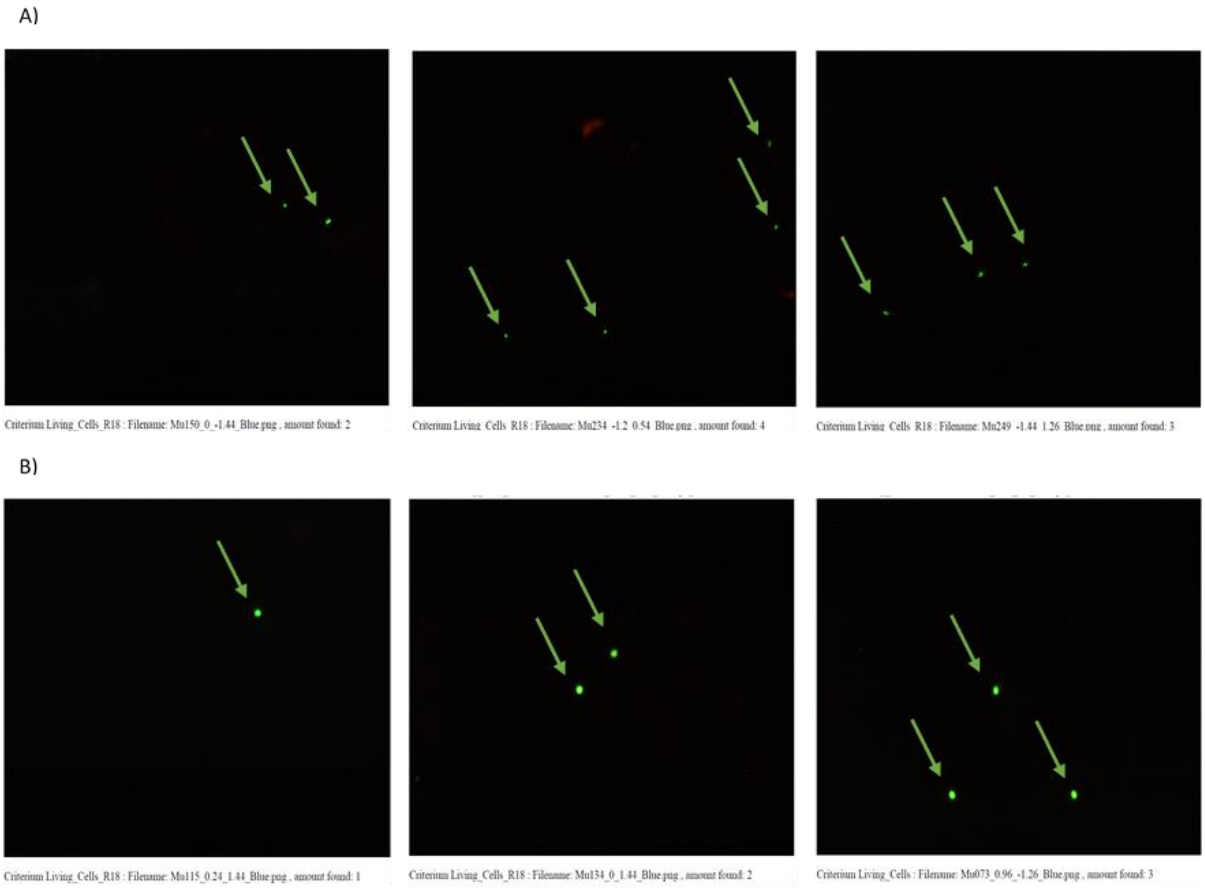

Figure 34. Scan images of *P. aeruginosa* (A) and *C. albicans* (B) obtained by solid-phase cytometry. Images shown correspond to sterile water inoculated with 10, 50 and 200 CFU/100 ml from left to right

3.2 Quantification of microorganisms in water for injection by solid-phase cytometry method

Water for injection (WFI) is supposed to have low microbial burden (less than 10 CFU/100 ml) as well as other physicochemical characteristics. It is expected that WFI hasn't any fluorescent component that could interfere with fluorescent viable cells. However, remains from the filtration steps it needs to undergo may be fluorescent and be detected by solid-phase cytometry. Moreover, these remains may even cause clogging of MuScan sieves, as the surface of it is significantly smaller than Milliflex membrane. The focus of this experiment was to test filterability of WFI through MuScan system as well as the presence of possible fluorescent interferences.

Water for injection was sampled from the company stream pipes and bottled in sterile recipients for transportation. Three samples of WFI were analyzed in parallel to 3 samples

of commercial sterile water and sterile PBS. Both sterile water and PBS were used as negative controls.

Firstly, 100 ml of WFI were added to the filtration system. Half of the volume passed in about 5 minutes, while the remaining 50 ml stacked and filtration stopped. In a second filtration, only 50 ml of WFI were filtered. Figure 35 shows scan images obtained when filtering 100 ml of WFI and filtering 50 ml of WFI. Fluorescent debris can be observed in the image corresponding to 100 ml of WFI, while no fluorescent background is observed when filtering only 50 ml. Filtration of more than 50 ml of WFI at once clogged the sieve.

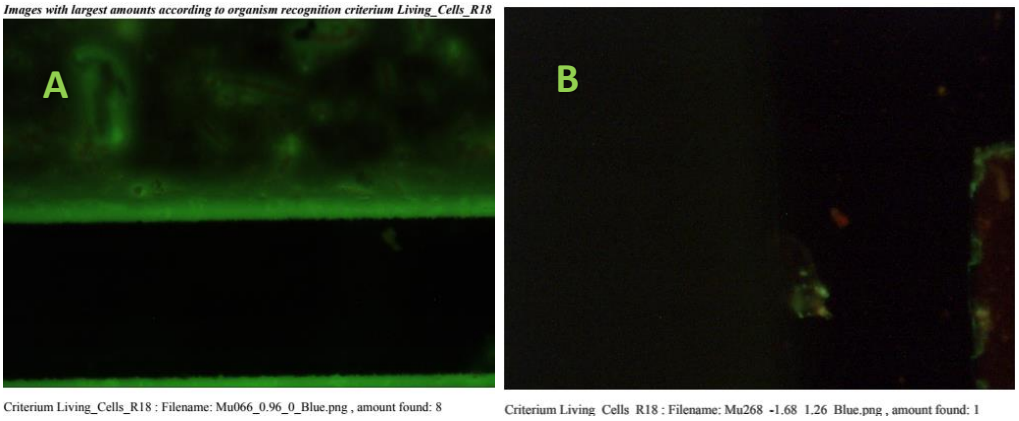

Figure 35. Scan images of 100 (A) and 50 (B) ml of filtered WFI

Secondly, three replicates of 50 ml of WFI and 100 ml of both sterile water and phosphate buffer solution (PBS) were assayed. Viable count for sterile water and PBS was in all cases 0. Viable counts in WFI was 0 in two of the replicates and 1 in the third replicate.

Figure 36 shows examples of scan images obtained after sterile water and WFI filtration. It has to be highlighted that 100 ml of sterile water were used while only 50 ml of WFI were filtered in this case, in order to avoid debris obtained with 100 ml. For that reason, results obtained with WFI need to be doubled in order to obtain the total viable count per 100 ml.

Scam images shown in figure 36 represent the ones with higher fluorescence. Viable cells weren't observed in sterile water replicates while one fluorescent structure was distinguished in WFI. The shape of the fluorescent spot can be attributed to a microorganism.

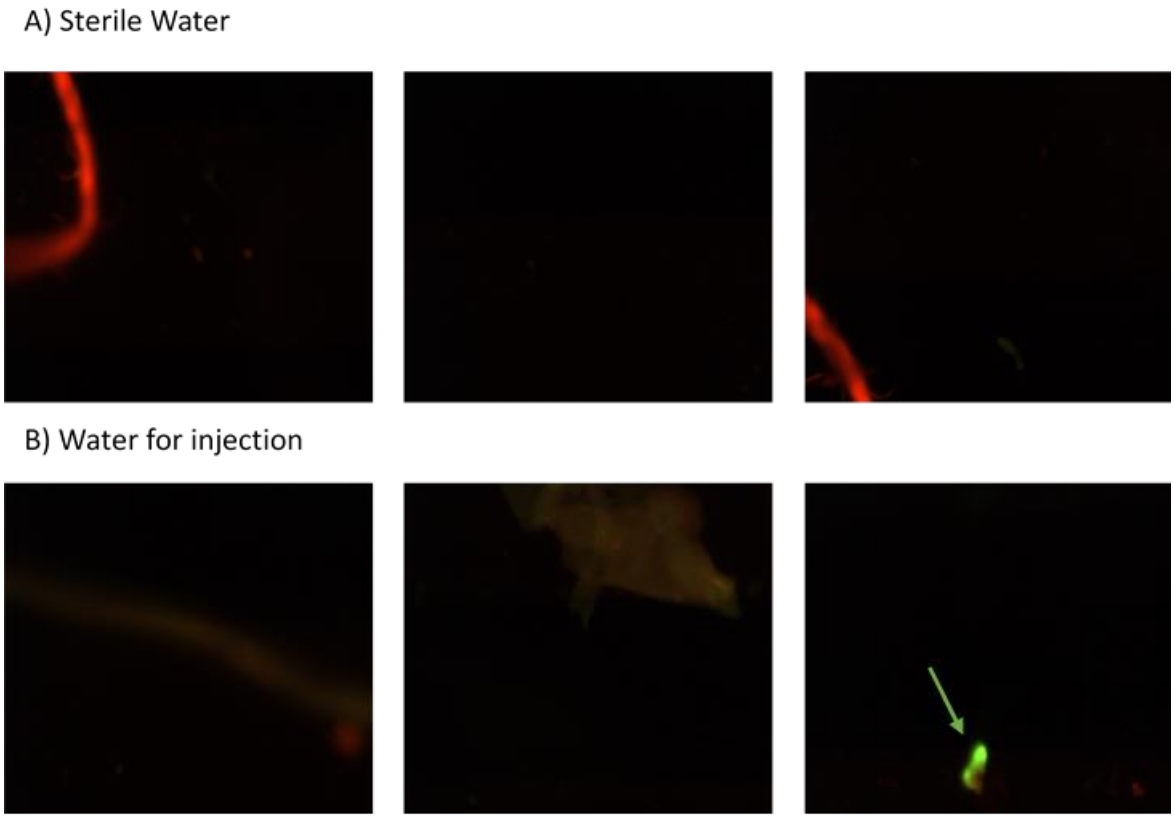

Figure 36. Scan images of filteres 100 ml of sterile water (A) and 50 ml of WFI (B)

3.3 Quantification of microorganisms in two different pharmaceutical products by solid-phase cytometry method

Total viable cell count is also used to assess bioburden of non-sterile products. Solid-phase cytometry system can also be used to test filterable pharmaceutical products. However, samples need to be tested for possible interferes before implementation of this technique.

Two products were chosen for testing the ability of MuScan to filter and detect viable cells present in them:

- A lyophilized antibiotic: Vancomycin
- An ear spray based on a saline solution

3.3.1 Assessment of bioburden of vancomycin with solid-phase cytometry system

Vancomycin is an injectable lyophilized product which needs to be at the end sterile. During its manufacturing process, bioburden of a bulk sample previous sterilizing filtration is performed as an internal control. For this, the objective of this assays has been to asses if solid-phase cytometry method is suitable for pre-sterilizing filtration bioburden. To do that, a batch of vancomycin from Reig Jofre was used.

In the first place, filterability of the sample through the sieve was tested. Filter surface from Millipore system is significantly larger than filter surface from MuScan sieves, so the last ones can be clogged more easily. A solution of 0.02 µg/ml was prepared and added progressively to the filtration system. MuScan filtration was performed in parallel to Milliflex filtration. It was determined that a vancomycin solution containing up to 0.2 µg of the product was well filtered. However, if solution exceeded 0.2 µg/100 ml of vancomycin the sieve was clogged and the solution remained stacked.

To test if vancomycin solution contained fluorescent interferes, three replicates of 100 ml containing 0.002 µg/ml of vancomycin were analyzed in parallel by MuScan and Milliflex traditional system. Sieves scanned in the solid-phase cytometry system showed mainly no fluorescence, except from 1 replicate that counted 5 viable cells. Plates from Milliflex filtration were incubated up to 3 days and any growth wasn't detected in any of the replicates. Scan images in which any fluorescence was detected are shown in panel A of figure 37. Five fluorescent structures were detected that resemble viable cells.

Additionally, in order to test if vancomycin inhibits detection of viable cells, a solution of vancomycin was inoculated with an overnight culture of *P. aeruginosa*. A volume corresponding to 150 cells/ml was added to the vancomycin solution. Two replicates of the solution were filtered by MuScan system in parallel with Milliflex system. Total viable count of the replicates obtained with MuScan was 1533 and 1503 respectively. Growth detected on plates was very abundant, so individual colonies were assessed as more than 1000 CFUs. Examples of scan images in which fluorescence was detected are shown in panel B of figure 37. Vancomycin did not inhibit detection of viable cells by solid-phase cytometry system.

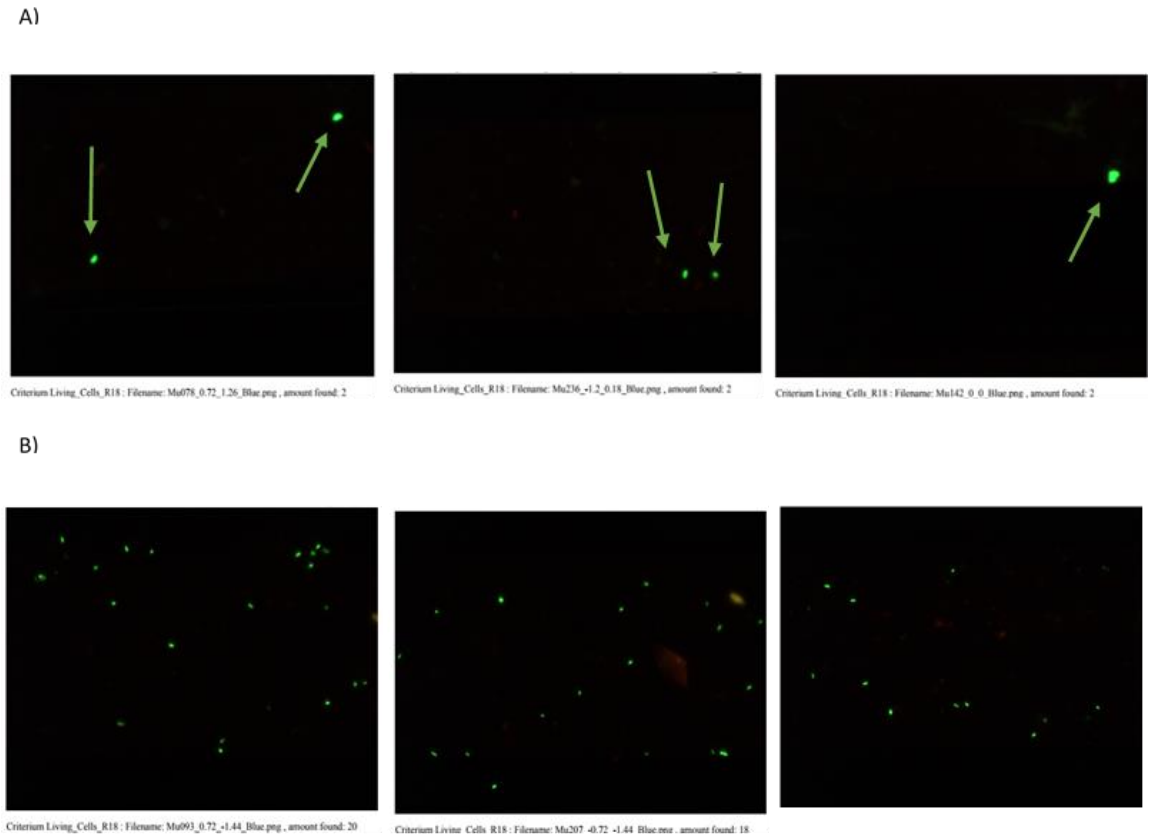

Figure 37. Scan images of vancomycin solution (A) and vancomycin solution with *P. aeruginosa* (B) by solid-phase cytometry method

3.3.2 Bioburden assessment of a non-sterile product by solid-phase cytometry method

In order to test applicability of the solid-phase cytometry system to non-sterile products, an ear spray cleaning solution was chosen. A batch of product that had previously determined to be contaminated by traditional methods was used for analysis with MuScan.

Filterability of the sample through the sieve was also tested for the ear spray. It was determined that 100 ml were easily filtered through the sieve without clogging the membrane. Three replicates of one hundred milliliters of the product consisting of a saline solution were filtered in parallel by MuScan system and Milliflex system. Total viable count results from MuScan were between thirty and sixty thousand cells per 100 ml. Plates from Milliflex system were observed after 24h incubation and already showed uncountable CFU/100 ml.

Figure 38 shows examples of scan images in which structured resembling a rod-shaped bacteria can be distinguished. Colonies observed in the plate present a blueish color, which is characteristic of *P. aeruginosa*. However, no further identification was performed as it wasn't the objective of the experiment.

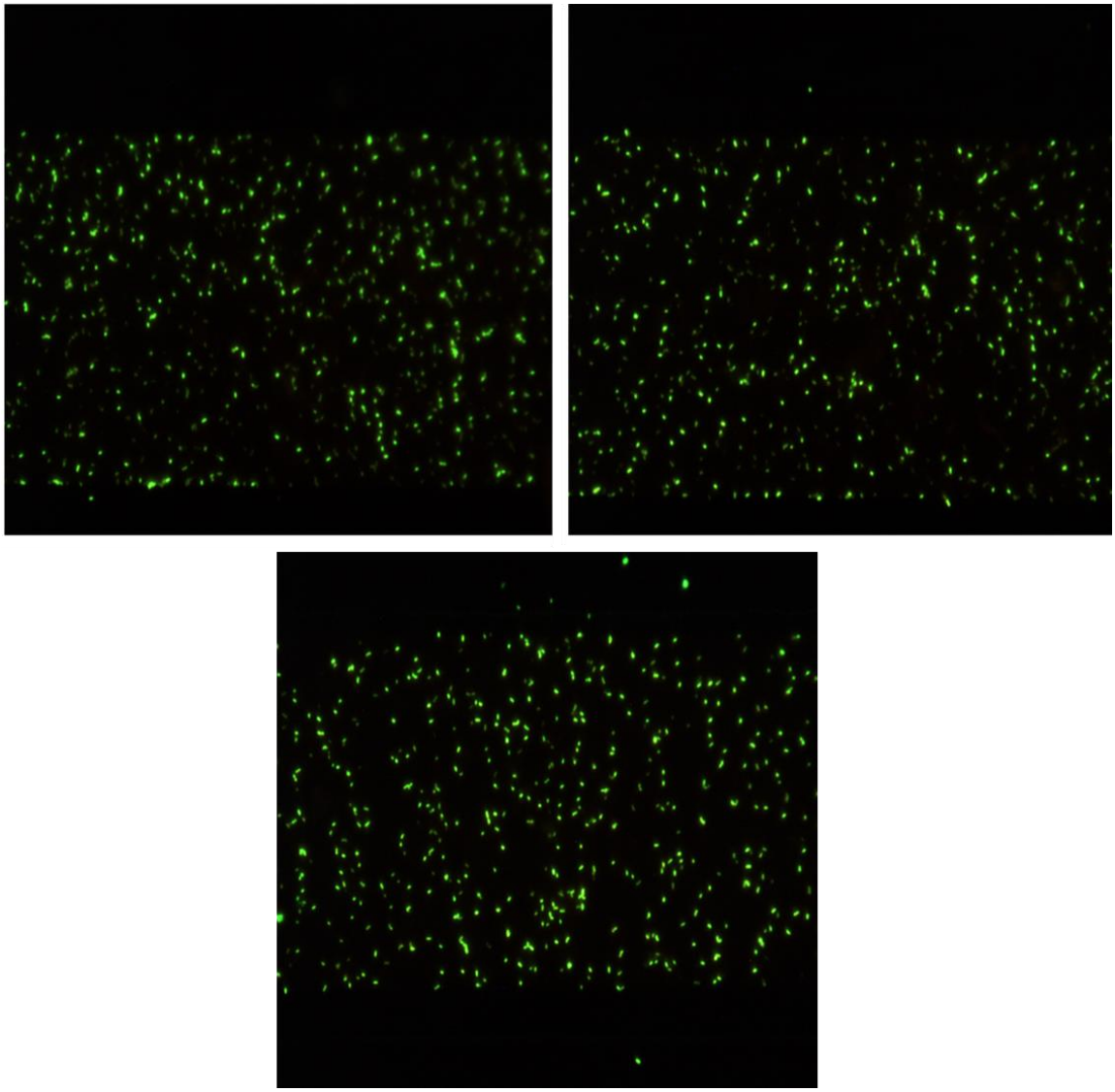

Figure 38. Scan images of three replicates of filtered ear spray solution by solid-phase cytometry method

Discussion

1 Summary of the implementation of RMM in Reig Jofre

Rapid microbiological methods can be applied to a diverse number of tests in the quality control of the manufacturing process and products. Taking into account that implementation of new methodologies is associated with an investment cost, priorities need to be defined regarding which parts of the process are in more need of technological improvement.

This project has focused on the implementation of RMM to the rapid and accurate identification of microorganisms as well as on the rapid detection of microorganisms in the environmental air and water. To do this, validation of every technique to be implemented was necessary, according to GMP requirements.

In the case of identification methods and detection of airborne microorganisms, an evaluation of the performance of both systems was carried out during a specific time period. In the case of solid-phase cytometry system for the detection of microorganisms in water and filterable products, the purpose was to perform pre-implementation tests.

Mainly, the project described in this thesis has lays the groundwork for implementation of RMM at different points of microbial monitoring of the manufacturing processes in the company. As a future perspective, rapid methods can also be seen as a step towards parametric release, having a higher control over the manufacturing process rather than relying on final product testing.

The different objectives initially proposed and experiments carried out for that purpose are discussed in more detail in the following lines. Benefits from implementation of RMM for the applications evaluated are exposed and future perspectives are discerned.

2 Establishing a microbial identification program: validation and implementation of identification methods

Microbial monitoring in the pharmaceutical industry involves pharmaceutical ingredients, in-process materials and manufacturing environments, in general. Identification of isolates recovered from the before mentioned activities is important in order to generate a microbial map and know which predominant species are found in the plant. This information can be very helpful when conducting investigations associated with product failure or loss of control over the process, for example in out of trend investigation in environmental monitoring.

In order to implement microbial identification methods in the manufacturing facility, an identification plan is designed that states the requirements for every isolate. According to the necessities of the company, a system or several systems are chosen and then validated for their routine use.

2.1 Definition of microbial identification program

Due to the high number of microbial monitoring activities performed to all materials and facilities involved in the manufacturing process, the number of isolates recovered every week can be large. Though, it is important to define which the critical points are, so that more detailed identification will be needed. This fact will in part determine the identification method of choice.

Pharmacopoiea guidelines point out that, on the one hand, isolates from media fills, sterility tests or microbial limits tests are the most critical, for which a species level identification will be needed. In addition, isolates found during any other activity that mean an out of specification or out of trend associated to an investigation are in the critical group.

On the other hand, for isolates from water systems analysis, pharmaceutical excipients or environmental monitoring, among others, identification at genus level will be acceptable. In these cases, alert and action limits are defined for every activity and just

microbial isolates trespassing limits threshold will be identified. For instance, in environmental monitoring, all microbial isolates from classes C and D won't need an identification, as they are the less critical areas in the manufacturing process.

2.2 Choice of an identification method: comparison of phenotypic and genotypic identification methods

Implementation of a new methodology in the pharmaceutical control environment needs a decision making process that is well established and documented. Many factors are taken into account from the science and technology-based characteristics that fit better operation needs as well as IT requirements and data integrity of the software components. For that, before making any purchase decision, a User Requirements Specification (URS) document was prepared with all the characteristics demanded for the system. A comparison of the available equipment and methodologies in the market was made, taking into account which system fits better the URS of the company and which system is less expensive for the company.

2.2.1 Technology and science-based differences

It is widely accepted in pharmaceutical references that genotypic methods are more accurate than phenotypic methods[85]. However, for routine identification, genotypic methods can be too expensive and laborious.

On their scientific basis, phenotypic methods rely on the microorganism phenotype, which is dependent on the culture media composition, growth temperature or other environmental conditions such as oxygen availability. In contrast, genotypic methods rely on nucleic acids that form the microorganism genotype, which comprises genes that encode proteins. Microbial genotype is relatively unstable, given the fast division rate of microorganisms gives rise to a high probability of mutation. However, genotypic identification methods such as PCR-Sequencing of *16S* and *18S* rely on conserved regions which don't vary from generation to generation. These genes are very useful for identification purposes given that they contain conserved regions that characterize them as universal and contain enough variability to discriminate between different

species. Taking into account those characteristics, PCR-Sequencing method is more accurate and reproducible than phenotypically-based methods.

However, phenotypic methods are still considered as first choice in many routine diagnostics laboratories because their easiness of use and non-expensive cost per sample. In addition, commercially available phenotypic methods have been developed to give consistent identifications in a routine use basis.

The main limitation of commercially available identification methods is the size of the database, which is a conditioning factor for the identification result. Generally, databases from phenotypic methods are smaller than those of genotypic methods[86], which makes phenotypic methods less accurate than genotypic ones and might bias the microbial map of a specific area towards the species present in the method database.

Commercially available identification methods have their own private databases. This is advantageous from the pharmaceutical regulatory point of view because they are already validated according to a series of standards. However, it is sometimes limiting as some of the isolates detected may not be present in the validated database.

One of the main constraints of using MALDI-TOF for microbial identification is that the database was originally developed for clinically relevant microorganisms. Regarding the results represented in the previous section, the majority of microorganisms isolated during microbial monitoring have environmental origin. However, MALDI-TOF is every time more used for environmental isolates identification[87,88].

Apart from technological and scientific considerations when choosing the method, GMP requirements must be taken into account. From this point of view, automated systems are preferred rather than manual ones. The advantage of phenotypic methods in front of genotypic methods is that the first ones have been automated for their routine use.

2.2.2 Initial investment and cost/sample of phenotypic and genotypic identification methods

The cost of the identification method was evaluated by taking into account the cost/sample and the time-to-result of the technique. An approximate number of 50

isolates per week was expected for identification, cost per sample and personnel time dedication of using genotypic methods was considered elevated for the quantity of samples to be processed[89]. In addition, isolates found in D/ISO 8/100,000 areas might only be characterized to the genus level, so genotypic identification is not necessary for all isolates found. Vitek-MS is a phenotypically-based method and cost/sample is very low compared to the rest of methods found in the market, sample preparation required few steps and personnel dedication and time-to-result is very fast.

When considering cost of an identification system, not only equipment cost is taken into account, as other factors such as validation cost, reagents, maintenance, etc. are considered. In addition, labor time and difficulty may impact on the cost of identifications, which is basically one of the major problems of genotypically-based methods for routine identification. The system based in PCR amplification and sequencing is very elaborate and this is why it is not considered as first choice for routine identification method, taking into account the number of samples per week. As not all the isolates require the same identification level, cost of genotypically-based methods may not always be justified. However, in the case of investigations derived from out of specifications in environmental monitoring or bioburden, cost is totally justified as determining the species or even the strain of a given microorganism may shed light into the origin of the contamination.

2.2.3 Implementation of the microbial identification program

The microbial identification program to be implemented in routine was the one represented in figure 24. As the schema shows, isolates from water systems, environmental monitoring or non-sterile pharmaceutical products will be identified by Vitek-MS method. Isolates from media fills, sterility tests and microbial limits tests will be identified by PCR-Sequencing method. In addition, failures from the Vitek-MS method will also be identified by PCR-Sequencing.

Identification method of choice for routine use was in the end Vitek-MS, which is a phenotypic system. This system based in MALDI-TOF has a sample throughput suitable for the need of identifying 50 samples per week, as it permits to identify 192 samples per run. In addition, personnel dedication time is lower compared to genotypically-based methods and samples are easily processed with few training.

However, knowing phenotypically-based systems weakness, a genotypically based method has also been validated for critical isolates and failures of MALDI-TOF identification method. The system implemented is not fully automated, as it is based on PCR amplification of *16S* or *18S* genes of bacteria and fungi respectively with universal primers, sequencing of the amplified region in an external sequencing service and data analysis performed back in the company. This last part is performed by a trained analyst, using NCBI public data base to achieve identification. This method required little initial investment for the company as no specific equipment is needed, apart from a thermal cycler which is a basic tool in molecular biology laboratories. Personnel time dedication and cost/sample is higher than that of phenotypically-based methods, however it is justified for the small amount of isolates identified by this system[86,90].

2.3 Validation of identification techniques

Identification methods to be implemented in routine were validated according to GMP. There is specific Pharmacopoeial guidance regarding validation of identification methods. Both EP and USP have similar indications on how to validate identification methods[91,83]. In our case, validation was performed to meet requirements of both Pharmacopoeia.

EP advises against comparing one identification method to another, as the ability to identify different microorganisms rely on how the identification is carried out and the different databases.

When validating genotypic methods, there is no gold standard with which to compare these methodologies to, as it is assumed that genotypic methods have the highest accuracy. For this reason, it was decided to validate both identification methods to be

implemented using reference microorganisms, preferably from the American Stock Culture Type Collection (ATCC).

Regarding validation results, MALDI-TOF based system and sequencing-based system perform very similarly in identifying microorganisms from ATCC strains. Accuracy and reproducibility have been assessed for both methods resulting in compliance for the parameters evaluated as it is shown in tables 8 and 9.

2.4 Performance of the implemented identification methods in the routine of microbiology control laboratory

Once the methods were validated, data obtained during their operation in routine was evaluated. Sets of internal isolates were used in order to compare MALDI-TOF and sequencing.

2.4.1 Comparison of MALDI-TOF and Sequencing methods in the identification of internal strains

Isolates stored in glycerol were recultured and transferred to a better preservation system at -80°C as explained previously. In addition, the recultured microorganisms were identified by sequencing and results were compared to previously obtained identifications by MALDI-TOF.

It is important to highlight that culture plates used for MALDI-TOF identification were timely different from the ones used for sequencing. MALDI-TOF identification was performed before storing isolates in glycerol while sequencing was performed several months later, after reculturing glycerol samples and transferring them to -80°C vials.

The fact that identifications weren't performed in parallel from the same samples can explain some of the differences observed in Table 9. MALDI-TOF and sequencing results agree at least at genus identification for the majority of isolates, as it was expected. However, 4 isolates had discrepancies at genus level identification. Isolate BACID32 was identified as *Bacillus infantis* by MALDI-TOF and as *Dermacoccus barathri*. by sequencing. Regarding the morphology observed by microscopy after Gram-Staining, images correspond to that of cocci, which indicates that recovery in the reculture was different from the original sample. The same explanation can be applied to isolates

BACID3 and BACID16, which were originally identified as *Corynebacterium* while recultured samples were identified as *Staphylococcus* and *Bacillus* respectively. Morphology of the Gram-stained recultured samples confirm a morphology compatible with *Staphylococcus* and *Bacillus* respectively.

The fourth isolate that arose discrepancy was BACID53, originally identified as *Ralstonia pickettii* and lately as *Moraxella spp*. Gram-stained sample shows a Gram-negative rod-shaped bacteria, which corresponds to the morphology of both genus of bacteria. However, the sequence obtained was more similar to that of *Moraxella*, which may indicate that the recultured microorganism was different from the original sample. Figure 39 shows partial fragments of the alignment obtained between BACID53 and *16S* sequences from GenBank of *Moraxella sp*. and *Ralstonia picketii*. In the regions shown, it is clearly observed that BACID53 sequence is more similar to that of *Moraxella sp*. than that of *R. picketii*.

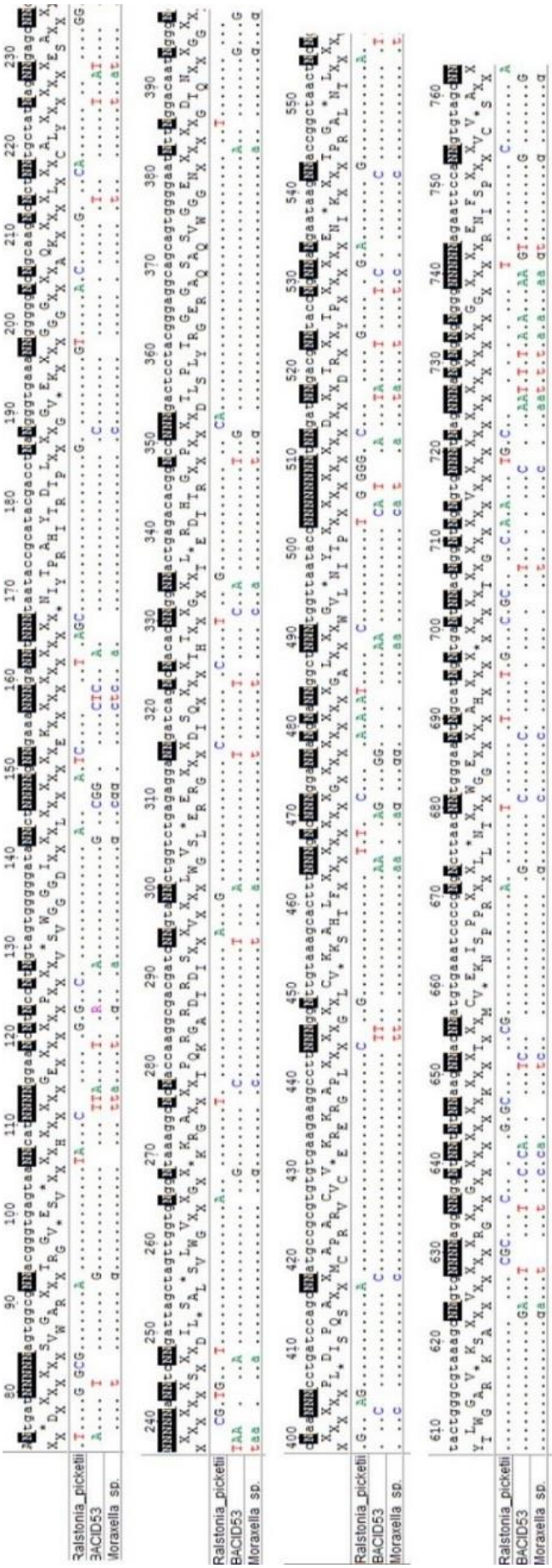

Figure 39. Alignement between 16S sequences of BACID53, Moraxella sp and R. picketii

In conclusion, MALDI-TOF and Sequencing give similar results, at least at genus level, which in most cases is enough characterization for isolates identified in pharmaceutical processes and products. Discrepancies at species level are consequence of taxonomical proximity and size of the database of identification method, which is not capable of detecting differences between taxonomically close microorganisms.

Discrepancies between methods that give rise to totally different microorganisms can be explained. Regarding the identification scores from each method shown in table 9, results for both methods were appropriate. So, it is highly probable that samples were contaminated when transferring the strains to glycerol. For that reason, when the strains were recultured for DNA-identification, a different strain from the original was obtained.

2.4.2 Comparison of Vitek-MS and PCR-Sequencing methods in the identification of isolates from environmental monitoring

Isolates recovered from environmental cleanroom air monitoring need to be identified. Air samples obtained by active air sampling or static sampling are incubated for 5 days at different temperatures: 2 days at 32.5±2.5ºC and 3 days at 22.5±2.5ºC. After this period, colonies are counted and isolated in pure cultures for identification.

To compare Vitek-MS and PCR-Sequencing a group of 46 isolates from environmental monitoring were identified by both methods.

Regarding the results in table 10, on the one hand, PCR-Sequencing method had 100% success in giving an identification result for every isolate. Species level identification was obtained for every isolate except in BZC25 and BZD45 and a percentage of identity higher than 90 was obtained for every identification.

On the other hand, Vitek-MS failed to identify 7 of the 46 isolates, which corresponds to an 85% success in giving an identification result. Failures correspond to genus *Globicatella*, *Brachybacterium*, *Kytococcus* or *Bacillus*, as they were identified by sequencing. In addition, Vitek-MS failed to identify 3 isolates that were identified as *Staphylococcus* by PCR-Sequencing. Species level identification was reached for every result, with a percentage of confidence higher than 99.

Regarding isolates that achieved an identification result by both methods, they coincide generally at genus and species. At genus level, all results except the one for BZD37 coincide. At species level, there are slight differences such as *M. luteus* or *M. yunannensis*, given by Vitek-MS and sequencing respectively for the same isolate. In addition, *S. caprae* or *S. capitis* are given for the same isolate by each of the identification method. However, these species are very close phylogenetically[92,93].

Regarding table 10, the majority of isolates correspond to Gram-positive bacteria, specifically cocci such as *Staphylococcus* and *Micrococcus*. These results aren't surprising given that only air samples are being taken into account for the comparison. Results are divided into isolates found in class C and class D cleanrooms and some differences are observed. For example, *Kytococcus* isolates were only found in class C while genus *Kocuria* isolates only appeared in class D. Other genus such as *Brachybacterium*, *Globicatella* or *Bacillus* have also been found in these analysis, although they appear punctually.

Figure 24 shows the number distribution of isolates in their corresponding genus and it is clearly stated that the two major groups are *Staphylococcus* and *Micrococcus* in a similar proportion. Fungi weren't detected during the course of this evaluation. However, they are found in some points of the microbial monitoring of products and processes although in a small proportion compared to bacteria.

2.4.3 Distribution of the microbial isolates in the manufacturing facilities

Knowing the microbial map of the manufacturing facility is essential for trend analysis and ensuring maintenance of control during aseptic processing. It is important to determine the most common microbial contaminants in order to establish an efficient and effective disinfection process.

The distribution obtained in the pie chart that constitutes figure 26 corresponds to a wide variety of microorganisms. It is worth to highlight that microbial isolates identified and represented here were found in the different microbial monitoring points of manufacturing process as well as raw materials and final products.

The most frequently found isolates correspond to Gram-positive cocci such as *Staphylococci* or *Micrococci*, whose major source is the environment as well as personnel. These microorganisms are most frequently detected in classified manufacturing areas of lower levels (C and D) present in the air or surfaces, as well as on personnel garments[94]. Results in table 11 confirm that the most frequently found microorganisms in environmental samples are gram-positive cocci. Regarding figure 27, gram-positive cocci mostly belong to two genus, so little diversity is found in this group that makes up 50% of the total isolates found.

The second most common group are Gram-negative rods, which are native of water sources, being the most commonly found genus *Ralstonia, Pseudomonas* and *Burkholderia*. This group constitutes an 11% of all the isolates found with diverse genus and species, although the most common ones are the above-mentioned. Some microorganisms forming this group are human pathogens, so especial care is taken to avoid this kind of microorganisms. A general study reported that the majority of product recalls due to microbial contamination are produced by gram-negative bacteria[95].

The third major group of microorganisms are Gram-positive spore forming rods, whose major representative is *Bacillus*. The ability of these organisms to develop spores helps them to survive in aseptic environments characterized by nutrient deprivation or lack of oxygen.

The greatest genus is by no doubt *Staphylococcus*, comprising 32% of the isolates, from which 17 different species can be found. Genus *Staphylococcus* has gained an interest focus because of *Methicilin Resistant Staphylococcus Aureus* (MRSA), which is a human pathogen that is resistant to a wide range of antibiotics. However, species of *Staphylococci* found in environmental samples are mainly non-pathogenic and autoctonous to human skin, such as S. *epidermidis*. In the same way, genus *Micrococcus* is directly related to *Staphylococcus* in environmental samples. The most abundant species of *Micrococcus* found is *M. luteus*, which is commonly found attached to dust particles and in the mammalian skin.

The third major group belongs to *Bacillus* genus, in a similar proportion to *Micrococcus*, comprising 9% of the total of isolates. *Bacillus* species are ubiquitous and diverse in

terrestrial and marine ecosystems, which facilitates their contamination of a variety of foods[96]. *Bacillus* individuals found in the pharmaceutical plant are mainly Gram-positive rods originating in environmental samples, such as agar-based active air sampling of cleanrooms or from microbial monitoring of personnel garments.

In addition to the three above-mentioned groups, genus *Kocuria* is always related to *Staphylococci* and *Micrococci* as a common environmental isolate. Some species of *Kocuria*, such as the most found *Kocuria rhizophila*, are commonly found in the soil which can be transferred to the manufacturing environment by personnel or material.

From water sources, Gram-negative rods such as *Pseudomonas*, *Ralstonia* or *Burkholderia* are the most commonly found genera[97]. In the isolates found in the manufacturing plant, they comprise nearly 26% of the total of isolates found.

According to Sandle 2015[97], *Pseudomonas* and *Ralstonia* seem to be more frequent in mains water system and purified water while *Burkholderia* appear more frequently in purified water (PW) and water for injection (WFI). By this means, it is apparent that genus *Burkholderia*, mainly *B. cepacia* and *B. contaminans*, are able to survive in harsh environments with low nutrient availability and extreme conditions, such as those of pharmaceutical use water. These water contaminants are specially taken care of because they are known to form biofilms in pipes[97] which are very difficult to remove.

The risk of the presence of *Ralstonia* in the pharmaceutical process is the relation of its presence with endotoxin generation, as the microorganism has the ability to pass through 0.2 µm filters, which are the ones used for primary sterilization[97]. In addition, gram-negative rods origin such as *Pseudomonas aeruginosa* origin nosocomial infections in immunocompromised patients[98].

From the results obtained it can be concluded that the major source of contamination in the manufacturing plant are personnel as well as the environment. Contamination can be introduced through the human skin as well as in dust associated to raw materials or equipment. The introduction of microbial contamination in the pharmaceutical process means a loss of control and probably failure in sanitation and decontamination. Knowing the major sources of microbial contamination helps to implement corrective

and preventive actions in order to avoid contamination. In addition, identification of contaminants aids in the effectiveness of cleaning and decontamination procedures.

According to the microbial identification program in figure 25, failures of Vitek-MS were identified by PCR-Sequencing. Table 12 shows only strains that aren't found in Vitek-MS database.

The majority of species that appear in the table correspond to Gram-negative rod-shaped bacteria, which are main contaminants of water sources. However, some of the microorganisms found in this group are commonly found in the environment, specifically in the soil, for example *Methylobacterium* or *Dyella*. Isolates from water of pharmaceutical use have been exposed to stressing conditions, so recovery of these organisms for identification is quite difficult. Moreover, as Vitek-MS database has been developed for clinical use mainly, some environmental species aren't present.

Regarding molds, genus identified by PCR-Sequencing are more varied than those identified by Vitek-MS. Taking into account results obtained by Vitek-MS, there are two predominant genus of molds: Aspergillus and *Penicillium*, although *Cladosporium*, *Fusarium, Rhizopus* and *Trichophyton* have been identified in a lesser extent. However, Vitek-MS has failed to identify *Acremoniun*, *Alternaria*, *Scopulariopsis* or *Trichoderma*.

For the remaining isolates, genus are common to both systems, although differences are found at species level. The majority of species that Vitek-MS fails to identify are due to the size and composition of the database (Vitek-MS database Saramis is smaller than that of the NCBI for nucleotide sequences). The size of the databases of different identification methods used in the pharmaceutical industry has been discussed before[86]. Moreover, as it has previously been discussed, MALDI-TOF database has traditionally been composed by clinically important microorganisms.

Regarding the few number of isolates that Vitek-MS has failed to identify compared to the total number of identified isolates, Vitek-MS is a suitable identification system for microbial monitoring. Nearly 90% of the isolates are identified by Vitek-MS, which is a non-expensive analysis and results are obtained in a short time period. Only few species aren't found in the MALDI-TOF database, which are successfully identified by sequencing.

3 A continuous laser-induced fluorescence system applied to the air monitoring of cleanrooms

The alternative rapid microbial method based on laser-induced fluorescence for the detection and quantification of microorganisms in cleanroom air has been qualified for its use in class A aseptic room. In addition, data from six months of operation of the system have been evaluated as well as the information provided from the alternative system in the investigation of excursions on plates from environmental monitoring.

3.1 Comparison of traditional agar-based active air sampling and alternative laser-induced fluorescence systems

This qualified rapid alternative method demonstrated to be appropriate for quantification of airborne microorganisms, and comparable in terms of microbial recovery to traditional methods per PMS validation[53]. Nevertheless, one of the reasons this alternative method cannot substitute the traditional method is that microorganisms cannot be recovered, preventing their identification and identification in class A aseptic rooms is required by both the European and US Pharmacopoeia.

Taking into account data from qualification of the system obtained in Reig Jofre facilities (in A, B, C and D classified areas), number of microorganisms recovered by the traditional and the alternative method is significantly different (table 13).

Laser-induced fluorescence system detected significantly a higher number of viable particles than CFU recovered by active air sampling and impaction on plates. The null-hypothesis for the equivalent test assumed that the systems were “Not equivalent”, so to test equivalence the result of the test must discredit the null hypothesis. After running the statistical test with data from table 13, the result was that the null hypothesis wasn’t discredited, meaning that data were statistically different. The p-value obtained with equivalence test was 0.951, so the alternative method cannot be considered statistically equivalent to the traditional method.

Data were standardized in order to get results from both system at the same scale and plotted in figure 29. Even standardized data shows differences between both systems and statistical equivalence cannot be demonstrated either.

Similar results were obtained by Miller and his group[51] when comparing an optical spectroscopy-based method (IMD-A technology-based BioVigilant) with an agar-based active air sampling methodology. The difference in the data may be explained firstly because the collection time for each system was different, although the volume of air collected by both systems was in the end the same. In that matters, laser-induced fluorescence system was exposed for a longer time to contaminants that may not have been detected by active air sampling system, which only was exposed to 10 minutes of air impaction.

In addition, regarding the technology of Biolaz and Sampl'air methods, differences in the data can be explained. Both systems are based on impaction sampling, however, collection efficiency and particle cut-off size of the surface of each system may vary from one system to another. Collection efficiency of portable microbial samplers has been assessed in previous studies[99], stating that collection efficiencies or active air samplers such as sampl'air are very low. Particle collection differences between agar-impaction samplers and laser-induced fluorescence methods might in part be explained by this reason.

Moreover, laser-induced fluorescence system detects viable but nonculturable (VBNC) microorganisms, which cannot be detected by agar-based active air sampling methods. Several authors state that the traditionally used system to assess microbial quality of cleanroorm air may underestimate the number of microorganisms present due to its inability to detect bacteria in this state[100,101]. Aseptic processing environments are susceptible locations for VBNC cells, as microorganisms are exposed to stressing conditions such as nutrient deprivation or physical and chemical damage.

VBNC microorganisms pose a risk that went unmitigated until the appearance of RMM. VBNC microorganisms are gaining importance in health systems as it has been proven that several human pathogens are capable of reaching this state when exposed to stress

conditions (such as those found in aseptic manufacturing) and then recovering its virulence when the stress factor is removed or favorable conditions are given[34,35,102].

As stated in the statistical results, the alternative method is more accurate than the traditional method in terms of quantification. In total, the laser-induced fluorescence system can be considered a supplementary method to the traditional one, as it provides real time information of the environmental state of cleanrooms.

Although equivalence between the traditional and the alternative method cannot be demonstrated, the alternative method can still be implemented in compliance with cGMP regulation. Organisms such as FDA encourage the use of rapid microbiological methods in the quality control of pharmaceutical products and processes and it is aware that the use of more accurate methods can give rise to a higher microbial recovery.

The existence of RMM has arisen the need to revise limits for environmental monitoring, as accuracy of less than 1 CFU can be achieved with these methods. Several studies have obtained similar results when comparing alternative methods with the traditional agar-based method [50,51,54] where the baseline of grade A rooms is different from zero. So, if the system has been properly validated for its intended use, it can be used in microbial monitoring in the pharmaceutical industry and from the regulatory perspective, there is high chance that specification in Annex I will change in the near future.

3.2 A risk-based approach for environmental monitoring

Once the equipment was qualified, it was installed in its final location. The laser-induced detection system was located in a class A room in the most critical point named I01, indicated in figure 40. Note that the system was qualified in parallel to sterile facility remodeling and requalification of cleanrooms. Requalification of cleanrooms implies the definition of microbiological sampling points, which is made in the basis of a risk assessment.

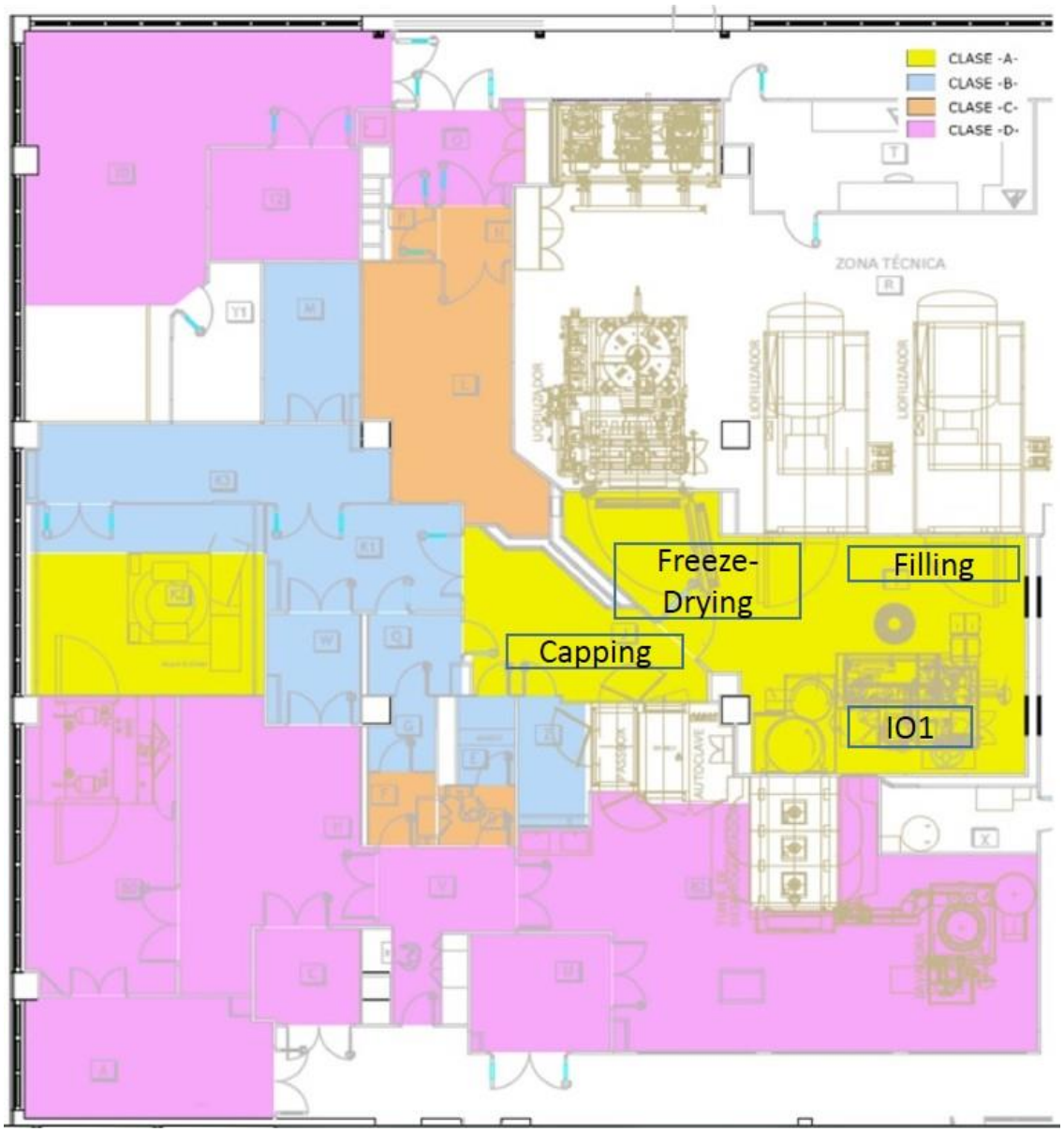

Figure 40. Schematic representation of the class A cleanroom in which the laser-induced fluorescence method is implemented

A risk assessment consists of evaluating the most probable sources of contamination in a facility in order to establish a higher control over them. There are different risk assessment tools[103], in the requalification of sterile facility clean rooms a parametric risk assessment was used. It consisted in assigning a risk potential from 1 to 4 to every activity that takes place, being 1 less critical and 4 the most critical one. Criticality is assessed of facility design, process and product exposure involved in every activity and the final potential is obtained with the following equation:

$$Risk\ potential = Facility\ potential\ \times Process\ Potential\ \times Product\ Potential$$

Parameters used to evaluate risk are shown in Annex II.

The number of critical points was determined according to the surface of each cleanroom, following UNE-EN ISO 14644-1:2015(E). Then, a risk potential was calculated for each point as explained before. Regarding the results obtained from the risk assessment, the most critical point for microbial monitoring was I01, which is located in a class A room in which filling takes place. During filling, product is exposed and it is introduced in its final container. For that reason, the laser-induced microbial detection system was located in this position. In addition, daily agar-based active air and passive sampling of air is taken in this same position.

Being I01 the most critical point in the aseptic process, a contamination found in this position means batch rejection unless absence of product exposure is properly justified. Nearly real-time data is extremely useful in this position because, when contamination is detected, the exact moment of exposure can be assessed by the number of biocounts detected at a given time. With this information and the register of activities performed in the room it can be assessed if the product has been exposed to contamination in order to avoid batch rejection.

From the risk management perspective, data from the rapid method will be used for contamination prevention upon the implementation of an action plan. Nearly real-time microbiological data permits to take mitigating actions when contamination is detected in the air in order to avoid product exposure. For risk prevention, an action plan needs to be defined and implemented.

3.3 Definition of action plan

One of the goals of the qualification of the alternative system for environmental monitoring of cleanrooms was the set of alert and action limits. Alert and action limits were calculated numerically on the basis of a statistical approach with internal data[6,13].

The action plan was defined after qualification and is the method by which corrective and preventive actions are implemented when excursions occur and it is intended as risk prevention. The action plan based on the alert and action limits calculated according to non-parametric tolerance limits approach will be the following:

- No action: 1 to 5 biocounts every 60 readings (10 minutes).
- Alert: 6 biocounts every 10 minutes = 6 biocounts every 60 readings. When detecting an alert, the event will be properly registered and documented and it may be used as extra information for sampling vials for sterility analysis of the product when required.
- Action: 9 biocounts every 10 minutes = 9 biocounts every 60 readings. This value can be used as an indicator of inappropriate conditions in the room, the moment when an excursion occurred or may have occurred. When the action level is reached, filling will stop automatically and vials present in the affected area will be removed from the chain. In order to restart filling, a minimum of 3 minutes (18 readings) with 0 biocounts after the event will be needed.

Establishing an action plan for a nearly real-time microbial monitoring system is a risk management approach. Traditional microbial monitoring of cleanrooms is based on incubation of plates and results are obtained after 3 or 5 days. Traditional microbial monitoring is not a risk management approach as action plan cannot be applied during manufacturing and if contamination is detected the whole batch of product needs to be rejected. Rapid microbial methods such as the laser-induced fluorescence system qualified for its use in environmental monitoring aids to take risk mitigating actions when contamination appears, avoiding rejecting whole batches of products.

3.4 Evaluation of laser-induced fluorescence system data during a six-month period

Once the alternative system was qualified on-site, it was set for operation in parallel to the traditional methods of passive air sampling (settle plates) and active-air sampling (slit-to-agar sampling). Environmental monitoring air sampling approach followed the dynamics represented in figure 28.

Although the action plan had been defined during the qualification of the system, it had not been implemented for the data evaluation period. Implementation of the action plan depends on several departments, from quality assurance to engineering to perform

different actions. In addition, personnel from the aseptic manufacturing facility needs to be trained before routine implementation of the new system.

Bearing results obtained during data evaluation shown in figure 31, an increase in the average biocounts/month is observed in September compared to the previous months. For that, biocounts produced during only filling operations in class A room during June and September were recovered and represented in figure 32. Regarding this figure, it can be observed that, even during filling periods, a higher number of readings above the action limit occurred in September when compared to June. This phenomenon can be explained as a result of the new lyophilization system beginning operation in the room during August. The change of room conditions affect flow of personnel and air so biocount recovery can be higher. However, regarding average biocounts from October to December, values decrease with time expecting them to stabilize at low levels. This phenomenon can be attributed to an adaptation process of the environment to the new conditions, most of all, personnel operation.

In order to determine that the state of the room has stabilized to low biocount levels, another six-month evaluation period will be needed once the equipment is reinstalled.

Taking into account the action plan proposed, different situations can occur when using traditional and alternative methods in parallel:

- Biolaz obtains a result over the action limit although growth in settle plates is negative. The part of vials affected in the time period when biocounts have been detected are removed as a preventive measure.
- Biolaz obtains a result over the action limit and there is growth in settle plates. Appropriate actions according to quality system are taken and if necessary batch is rejected.
- Biolaz results are below the action limit and growth in settle plates is negative. The batch is fully approved for release.

In terms of the economic benefit in implementing a laser-induced fluorescence system for environmental monitoring, there are expected long-term savings. This is because knowing the exact moment a contamination occurs, allows the affected part of the batch to be separated from the total lot. As part of the action plan, when action limits are

reached, vials affected in the moment of excursion can be set apart from the batch being filled, removing that risk immediately. It is possible to know when to restart filling as the room will be at 0 counts for the following 18 readings, ensuring the contamination has been removed and manufacturing can continue safely.

3.5 Benefits of the action plan of laser-induced fluorescence system

One of the main benefits of a laser-induced fluorescence system is that it allows a high level of control over the process due to emitting data in nearly real time. It is not possible to remove settle plates from environmental monitoring program as it is a requirement for batch release, but the alternative method informs about the state of the room. It is expected that new Annex 1[6] will suggest the use of alternative methods, not with the intention of replacing settle plates, but to complement them. System data enables the anticipation and detection of risk the moment it occurs, which helps personnel take immediate action and prevent consequent contamination. In addition, when excursions in plate counts happen, information provided by rapid alternative methods help in the investigation of the batch in quarantine. For Quality Assurance purposes, real-time data aids in determining whether a serious contamination is present in the air during filling, or if contamination was produced during, for example, preparation or maintenance. Risk management can be used to facilitate improvement of the final product quality and safety.

It has been proven that accuracy of alternative methods is higher than that of settle plates, and settle plates, as a method, cannot be validated. So, with proper validation and qualification of alternative systems such as a laser-induced fluorescence detection, new limits for routine monitoring can be set up instead of the typical CFU/m^3. This rapid method is not capable of recovering and identifying microorganisms detected, so active air sampling will still be needed. However, as action is taken at the moment contamination occurs, identification is not as critical as in settle plates, where results are obtained five days after contamination occurs.

In conclusion, the laser-induced fluorescence detection method for environmental monitoring has proven to be more accurate than the traditional system and the fast time-to-result permits taking immediate action when limits of contamination are

reached. The future of quality control in microbiology is moving towards implementation of alternative technologies. The initial effort in validation will translate into economic savings for the company and, most importantly, increased quality and safety of the products.

4 A rapid microbiological method for the quantification of microorganisms in filterable samples: water for pharmaceutical use and products

The third general objective of this project was to implement a rapid microbiological method for detection and quantification of microorganisms in filterable samples. Filterable samples enclose from water of pharmaceutical use to the majority of products manufactured in RJF: from liquid injectable to lyophilized products.

4.1 Choice of the rapid method for implementation study

Criteria to choose a rapid method to quantify microorganisms in filterable samples were fast time-to-result result, more sensitivity than the traditional system and non-growth based. In addition, it must be in agreement with GMP regulation, which means that it is susceptible of validation and the software must accomplish 21 CFR part 11 of GMP guidelines.

In the previous phase to testing, different methods available were evaluated, whose scientific background is explained in the introduction part of this text. For example, ATP-Bioluminescence based systems such as Celsis® or Milliflex were considered for implementation. These systems can be easily validated because their technology is based on detecting the same which is detected with traditional methods, as they are growth based although faster. However, as incubation is still needed and the will of the company was to invest in a more innovating technology, these systems were discarded.

Table 16 shows the different options evaluated and compared for its implementation in RJF. Characteristics used for comparison are shown in the table, being the most important at the moment the time-to-result. On that basis, MuScan was chosen for

experimental evaluation and future implementation in the control of the aseptic process and products.

Table 16. Comparison of available rapid microbiological methods evaluated

	Method Applications	Time to Result	Sample Size or Type	Sample Treatment	Destructive/Non destructive	Sensitivity	Regulation acceptance	Organisms Detected
Celsis Advance System using AMPiScreen	Enzyme-amplification combined with ATP bioluminescence	2-6 days (Sterility)	User selected	Sample treatment is the same as in traditional method	Destructive	1 CFU in pre-enriched sample	21 CFR	Bacteria, yeast, mold
LumiByte BV MuScan /Innosieve Diagnostics	Viability staining and solid phase cytometry; fluorescence microscopy	65 minutes	Filterable samples; 1 µL to 1L	Special filters used that enable later laser detection on the same filter.	Destructive	1 - 10^5 cells	21 CFR in progress	Bacteria, yeast, fungal spores
Rapid Micro Biosystems / The Growth Direct™ System	Cellular auto-florescence	7 days	Filterable samples	Totally new filtration system and self-culture media.	Non-destructive	1 CFU	21 CFR	Bacteria, yeast, mold, all organisms that grow on agar media
Merck – Milliflex Rapid Microbiology	ATP bioluminescence in plate	Results in approx. 5 days	All filterable samples	Millipore filtration System.	Non-destructive	1 CFU	21 CFR	Bacteria, yeast and molds

Solid-phase cytometry based system doesn't need an incubation prior to detection because it has a sensitivity of 1 cell. In addition, improvements on imaging software analysis permit an automatic counting of viable cells, although trained personnel can discriminate between viable cells or debris basing on microbiological background. This system, commercially known as MuScan, permits to have results in approximately 2 hours, which makes it a very practical solution for testing water for injection and knowing the result before manufacturing process takes place.

4.2 Application of solid-phase cytometry system to water analysis

Results obtained show that the rapid method quantitative capability is similar to that of the traditional method. Note that the sensitivity of the alternative method is 1 cell and results are obtained in cell numbers while the traditional method detects CFU. An assumption is made that 1 cell originates 1 CFU. This fact can give rise to differences in the recovery of microorganisms as seen in table 10.

The experiments carried out with water for injection (WFI) showed that there weren't mainly fluorescent interferes. Some synthetic fibers or residues from the production of WFI might be detected with the solid-phase cytometry method. This could explain the fluorescent background obtained when filtering 100 ml of WFI at once, while 50 ml of WFI doesn't show this fluorescence.

The main disadvantage of MuScan with respect to the traditional method is that membrane can clog more easily than Milliflex membranes. The Sieve® membrane has been enhanced so that the surface is as flat as possible in order for the imaging scan to cover all of it. However, the surface of the membrane is very small compared to the Milliflex one, so that the product concentrates in a smaller space lowering filtration and even clogging so that filtration stops.

This phenomenon could be solved by dividing the volume necessary for filtration according to Pharmacopoeia in several sieves and adding results of individual sieves at the end of the scan process. Time-to-result wouldn't be significantly affected because several membranes can be processed at a time, however the cost per sample would significantly increase.

4.3 Application of solid-phase cytometry to bioburden of products

Results with the different products tested show that there aren't fluorescent interferes present in the product's matrices. In addition, when testing antibiotic vancomycin, there is no inhibition of microbial growth so contamination could be detected with this alternative system.

Comparing filtration of both products tested, vancomycin and ear spray, the last one shows an easier and faster filtration. Quantities of vancomycin higher than 0.2 µg can clog the sieve and stop filtration. The same approach described for WFI could be adopted for product bioburden: dividing the sampled volume indicated by Pharmacopoeia in different sieves and adding the results of the individual sieves for the result of one sample. In the case of vancomycin, more than 5 sieves will be necessary for filtrating the necessary volume indicated in the protocols. This means a significant increase in the cost/sample, as Sieve-ID method is per se more expensive than Milliflex method and several analysis need to be performed in order to filter the same volume.

4.4 Viability of implementation of the solid-phase cytometry method in the process control of pharmaceutical manufacturing

The solid-phase cytometry method has demonstrated to be fast and effective for microbial detection and count. The fact that it isn't growth-based allows having results in less than 24 hours. The characteristics that define this rapid method make it very interesting for application to in-process testing during manufacture.

4.4.1 Application of RMM in the manufacturing process

Firstly, a possible application of the method would be in the analysis of WFI before mixing with raw materials. Regarding figure 2 from the introduction section, mixing step is one of the firsts in the process. If solid-phase cytometry method was applied in the daily analysis of water the state of it and its suitability for manufacturing would be tested in a timely manner. Water testing with traditional methods has a time-to-result between 3 or 5 days, which implies that if the water was contaminated it will be detected once

the product is already in its final packaging. Testing with traditional methods isn't a risk management approach, while testing with the rapid method allows implementing Corrective and Preventive actions (CAPA) in order to avoid manufacturing a contaminated product.

Secondly, another application of this technique will be the bioburden determination before sterilization. Bearing in mind figure 2 from the introduction, it's the step between dissolution and filling. There are several forms to perform sterilization, in Reig Jofre manufacturing facility the majority of sterile products manufactured undergo a sterilizing filtration and aseptic filling. It is required by Pharmacopoeia that bioburden in this point is ensured minimum so sterilization is effective. With the solid-phase cytometry method results will be obtained in more or less two hours, while other preparation activities take place, knowing the state of the process in a contemporary manner and having the possibility to implement CAPA in case contamination is detected.

Finally, this rapid method could be applied to final product testing, for example bioburden of non-sterile products. Results will be obtained faster, decreasing the release time necessary for all quality control tests to be performed. However, this step is not as critical as the before-mentioned ones. It is advantageous to obtain faster results in microbiology, as they commonly are the longest compared to physical and chemical testing

4.4.2 Cost of implementing the solid-phase cytometry method

The cost of the system to be implemented needs to be taken into account when implementing a rapid method. In a general basis, there is an initial investment and cost/sample in disposable materials and reagents.

In order to approximately calculate the return of investment (ROI) of this rapid method, firstly the number of tests to be performed with the rapid system needs to be assessed. Water testing of both purified water and WFI means approximately 2000 tests in a year. Taking into account that cost per sample with MuScan is nearly 4 times the cost of Milliflex system, the increase in budget is considerable. Personnel time dedication is approximately the same for both systems.

The number of product batches rejected because of water contamination determines the ROI for this method. If losses related to batch rejection are higher than the cost of the system, then the investment will be economically worthy. However, batch rejection due to water contamination isn't a major problem in RJF manufacturing facility at the moment.

4.4.3 Constraints of solid-phase cytometry

One of the main disadvantages of this method is that microorganisms retained in the sieve are rarely recovered after staining. Recovery of microorganisms after detection is necessary for the further identification of the same.

With sieve-ID technology and other solid-phase cytometry-based systems, cells are stained with a viability marker for their detection. The viability marker sometimes permeates the cell and kills it or stress it in a way that it cannot grow in culture media. For the majority of the isolates recovered in the microbiology control of processes and products in the pharmaceutical industry, identification is mandatory. So recovery of the microorganisms detected is essential.

This constraint with solid-phase cytometry systems can be solved by performing in parallel a Milliflex filtration and incubation of the filter. For that, the sample should be divided, which in addition implies to double sampling for testing the same quantity with both methods as Pharmacopoeia indicates the exact volume needed for testing. In this way, the numerical result will be obtained in a few minutes while filters will follow an incubation period until enough cellular mass is obtained for identification.

This fact increases the cost of the analysis and the sampling volume needed, which is necessary to take into account when calculating the return of investment (ROI) of the alternative system. In addition, the assumption that microorganisms are distributed homogenously in the fluid is made, so that approximately the same microorganisms will be recovered in the same volume by both methods. However, strictly speaking, it is not exactly the same sample, so differences can be found between recoveries from both methods

5 Implementation strategy of RMM in the company

With the results presented in this project, the strategy followed to implement RMM in the control of manufacturing process and products can be summed up in the following diagram (figure 41):

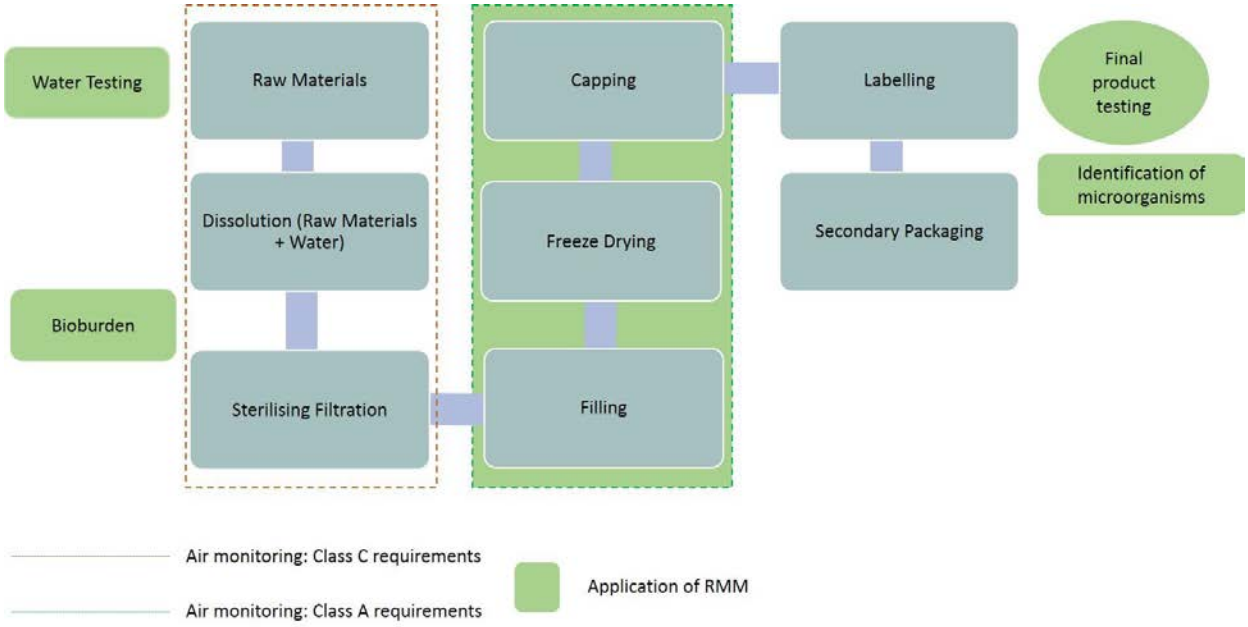

Figure 41. Schematic representation of the RMM implementation strategy in the company

Regarding figure 41, the activities in which RMM have been applied are highlighted with green background. A rapid method would be implemented for the quantification of microorganisms in WFI as a raw material, knowing before mixing if it is microbiologically suited for manufacturing. The following step in which RMM would intervene is bioburden of the bulk product before sterilizing filtration, which is a necessary step in the manufacture of sterile pharmaceuticals. Results would be obtained in a timely manner so a higher control over the process is gained.

Activities such as filling, freeze drying and capping take place in an aseptic zone as the already sterilized product is exposed to the environment. Air monitoring of this zone with class A requirements will be performed with a rapid method that allows to have results in a timely manner (a reading every 10 seconds) which is nearly real time.

Finally, implementation of rapid identification methods support all of the manufacturing process, as microbial isolates can be found at different points. In a global, this RMM implementation strategy allows a higher control over the process and follows a risk management approach, in which preventive actions can be adopted.

6 Assurance of microbial control along the manufacturing process

The strategy described in the previous section consists of an approach to gain control over the process, whose final objective is ensuring sterility of the final product.

Sterility of a product is defined as absence of viable microorganisms while viability is defined by microbiologists as the capacity of microorganisms to form progeny[102]. In a practical way, viable microorganisms have always been assessed as the ones capable to grow in culture media. However, there is a vast number of species that cannot be cultured. The presence of VBNC microorganisms is accepted and it is known that poses a risk in sterile manufactured products. Traditional methods in microbiology aren't capable of detecting VBNC microorganisms, so microbial flora in the pharmaceutical environments is usually underestimated.

Sterile pharmaceuticals need to undergo sterility test stated by Pharmacopoeia. Nevertheless, limitations of the sterility test are well known and have been discussed [17,104]. The probability of finding contamination if any is very small due to the sample size used for the test, EP and USP allow a maximum of 20 units for a batch of 500 units. Only in conditions of heavy contamination sterility test will be positive and even in those conditions only microorganisms able to grow in culture media will be detected[105].

Regarding pharmaceutical products and according to guidelines on sterile batch release, sterility test as described by Pharmacopoeia must be accomplished but it doesn't demonstrate sterility of the whole product. A successful result in sterility test means the absence of microbial growth in the conditions of the test and for the amount of product that has been tested. Because of that, sterility of the product must be assured along the manufacturing process.

Sterility assurance concerns the wider aspects of Good Manufacturing practices, which are designed to protect the product at all stages of manufacturing (from incoming raw

materials through to finished products) and thus it forms an integral part of the quality assurance system[105]. Rapid microbiological methods can play an important role in maintaining control over the process because of their higher accuracy in comparison to traditional methods. In addition, rapid methods that don't depend on growth for microbial detection are capable of detecting VBNC microorganisms, giving a more realistic assessment of microbial flora.

7 Future perspectives for rapid microbiological methods and the sterile manufacturing facilities

Technological advances have facilitated the development of more accurate and faster methods such as RMM. The pharmaceutical industry is characterized by a conservative attitude reluctant to changes. However, the patient's safety is in the end the essential goal of cGMPs and technological advances help pharmaceutical industries to move forward in this direction. Assurance of microbiological safety of pharmaceuticals can benefit from technological advances and more companies are adopting them to enhance the quality of their products.

7.1 The role of RMM in QbD and PAT

The Process Analytical Technology (PAT) is defined as a system for designing, analyzing and controlling manufacturing through timely measurements (during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of improving the product quality[18]. The PAT implementation is an initiative of the FDA that has born as a measure to implement QbD in the pharmaceutical manufacturing process[106,107].

The first step in PAT implementation is the design phase in which a risk assessment is made[108,109]. Regarding microbiological control, the most critical points in the aseptic process are measuring bioburden before the filter sterilization step and environmental monitoring during the aseptic filling process. Pre-sterilization bioburden level is critical because a high microbial burden can overload the filter and acceptable levels of endotoxins in the final product need to be ensured. Environmental monitoring of the

aseptic filling step is critical because the product is exposed to the environment and with actual traditional microbiological methods results are obtained several days after the activity[110].

Considering that PAT relies on timely measurements of in-process materials and corrective action in real-time, the RMM evaluated previously are an approach towards this end. The laser-induced fluorescence system Biolaz is a true PAT solution whose results are obtained in a timely manner and allows risk management during the manufacturing process. Pharmaceutical microbiology experts such as Miller or Sandle have described optical spectroscopy-based methods as an approach to PAT and the future of microbiology testing in pharmaceutical manufacturing[50,111].

Regarding solid-phase cytometry, results aren't obtained as fast as in optical spectroscopy. However, they still are obtained in a timely manner. If applied to bioburden of the pre-filter sterilization step, results can be obtained in a maximum of two hours and with the proper planning of activities, bioburden levels of the bulk material could be known before filtering.

From the microbiology control perspective, the pharmaceutical industry can benefit from QbD and PAT principles to:

- Design robust processes that prevent contamination.
- Ensure that a state of microbial control is maintained.
- Develop more effective strategies to correct a contamination problem.
- Continually improve processes and products.
- Assess the potential impact of failing results on the patients[106].

One of the pursued consequences of implementing PAT is parametric release of products, eliminating the necessity of final product testing. From the microbiology point of view, parametric release can only be applied to finally sterilized products and not to aseptically manufactured ones[105]. However, with the increasing availability of real-time in-process RMM a new scientific and regulatory framework might be provided for implementing parametric release even in aseptically-filled products[106]. Nevertheless, this still is an optimistic future perspective although every time regulatory organisms are more prone to changes on the basis of scientific evidence[112].

As a summary, the implementation of next generation of rapid microbiological methods represents significant progress toward the acceptance of microbiological PAT solutions for the industry and is directly aligned with the expectations for pharmaceutical manufacturing, quality and operational excellence of the 21st century.

7.2 Future perspectives on sterile manufacturing processes

New technologies are being developed in order to minimize human intervention in the manufacturing process of pharmaceuticals, as one of the main sources of contamination. These technologies include equipment automation, barriers and isolator systems. In these types of facility where personnel have been completely excluded from the critical zone, the necessity for room classification according to particle content and microbiological environment requirements may be significantly reduced.

These kind of facilities in which human intervention is nearly absent require a high level of automatization. In this context, RMM offer the sensitivity and automatization level demanded.

The project described in this dissertation lays the groundwork for implementation of RMM at different points of the sterile manufacturing process and final products. In a recent future, a new sterile manufacturing line will be set up in RJF manufacturing plant, in which human intervention in the aseptic process is completely eliminated. Sterility will be maintained and controlled with the use of isolators, in which optical spectroscopy-based methods in combination with dynamic sampling agar-based methods will be used for microbial environmental monitoring.

In the same way, other RMM could be applied to in-process testing, to reach the goal of timely testing for QbD and PAT purposes, as described previously.

The future of sterile manufacturing seems to be fully automated processes in which real-time data is obtained, even microbiological quality assessment. The final goal of this approach is to have every time more control over the process in order to increase product quality and safety, to minimize the patient's risk.

Conclusions

In relation to the objectives presented at the beginning of this thesis, the following conclusions can be drawn:

1. A microbial identification program based on Vitek-MS and PCR-Sequencing has successfully been implemented and both methods have been validated according to cGMP.
2. Vitek-MS method is appropriate for the identification of the majority of the isolates found in the manufacturing environment and products as its success rate of identification is 87%.
3. PCR-Sequencing method fits as the back-up microbial identification system as it successfully identifies isolates failed by Vitek-MS.
4. A RMM based on laser-induced fluorescence has successfully been qualified for viable particles monitoring in cleanroom air.
5. The laser-induced fluorescence system is more accurate and sensitive, which arises great differences with the traditional slit-to-agar method. For this reason, equivalence between the laser-induced fluorescence method and the traditional slit-to-agar method cannot be statistically demonstrated.
6. Alert and action limits have been defined for the alternative method in base to a non-parametric tolerance-limits approach. These limits were used to establish an action plan for the detection of air contamination in nearly real time.
7. The laser-induced fluorescence method works as a risk management tool in order to be able to prevent contamination and mitigate risk in the same moment it is detected.
8. The applicability of a RMM based on solid-phase cytometry has been tested with water for pharmaceutical purposes and filterable products determining that the method is suitable for its use in the tested products.
9. The solid-phase cytometry system is suitable for bioburden of water, vancomycin and ear spray products as no fluorescent interferes are produced.
10. The solid-phase cytometry method applied for water and pre-sterilization bioburden of products testing will allow to prevent manufacturing of contaminated products as results are obtained in a timely manner.

11. RMM used in this thesis have proven to be faster and more accurate than the traditional microbiological methods. Implementation of RMM is beneficial for the pharmaceutical companies in that products can be released faster, which in the end can translate in economic savings despite the initial investment. Finally and most important, the increased accuracy of RMM allows a better knowledge of the microbiological content of products and processes which translate in higher quality and safety for the patients.

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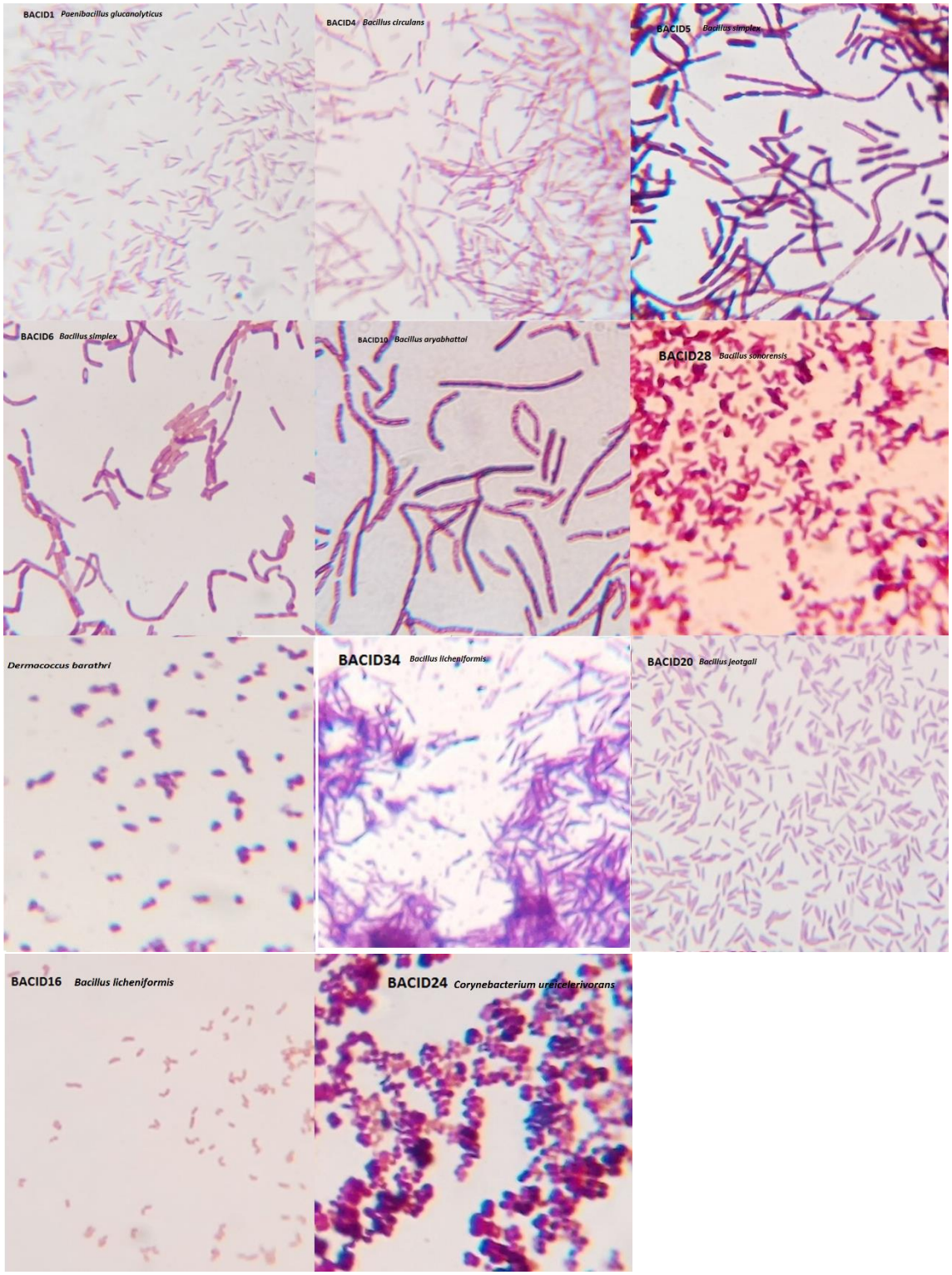
Annexes

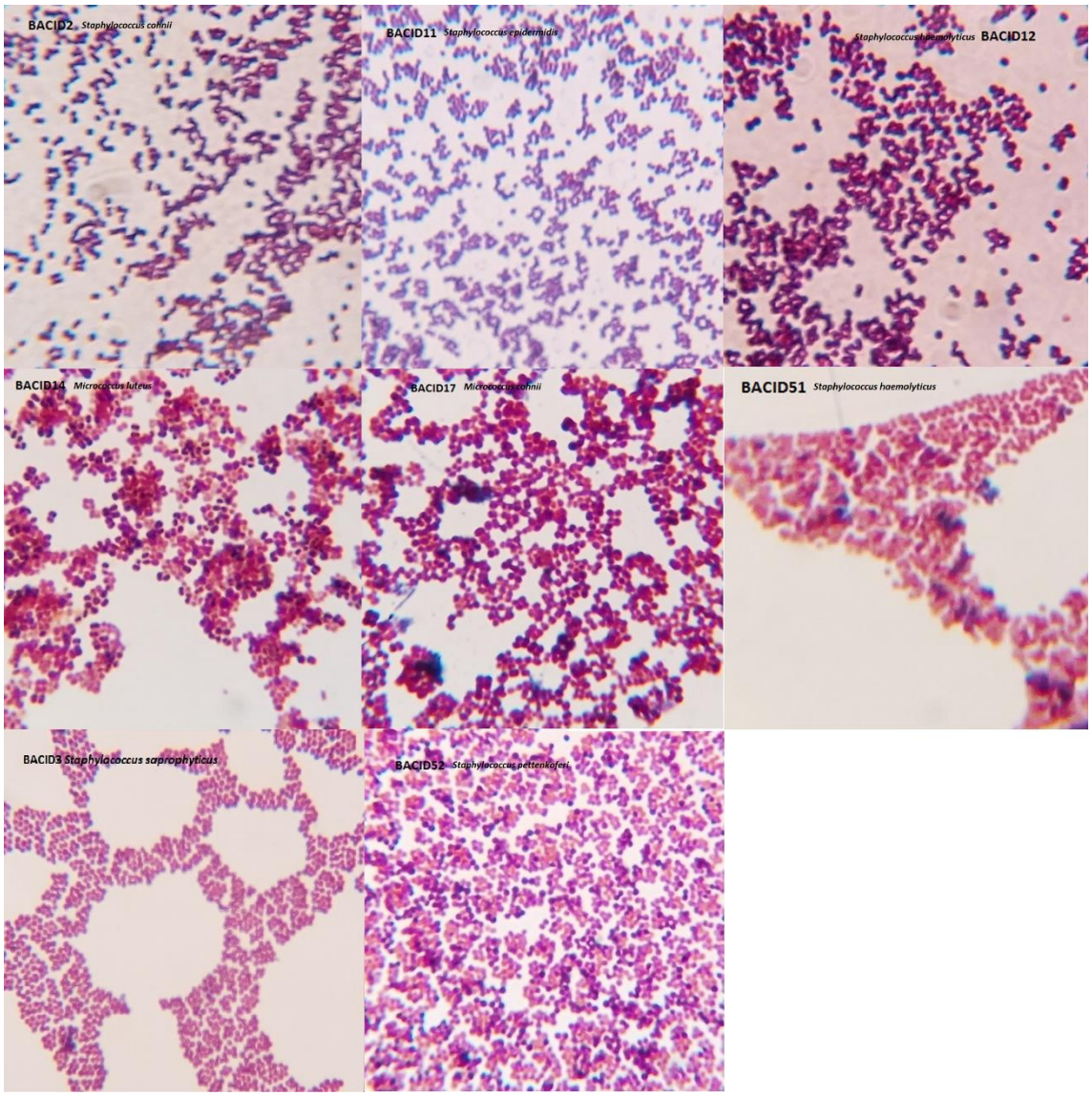
I. Microscopy images of in-house microbial isolates identified by PCR-Sequencing

A) Gram-positive rods



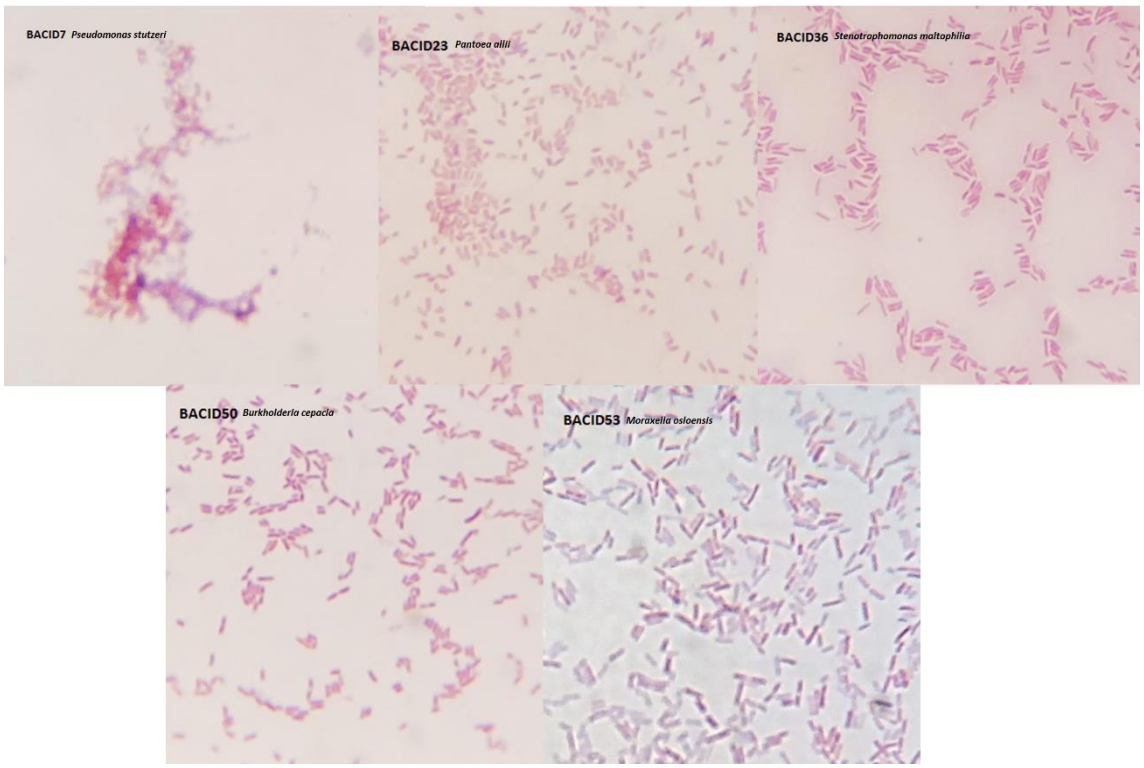
Annex Figure 1 Gram-positive rods

B) Gram-positive cocci



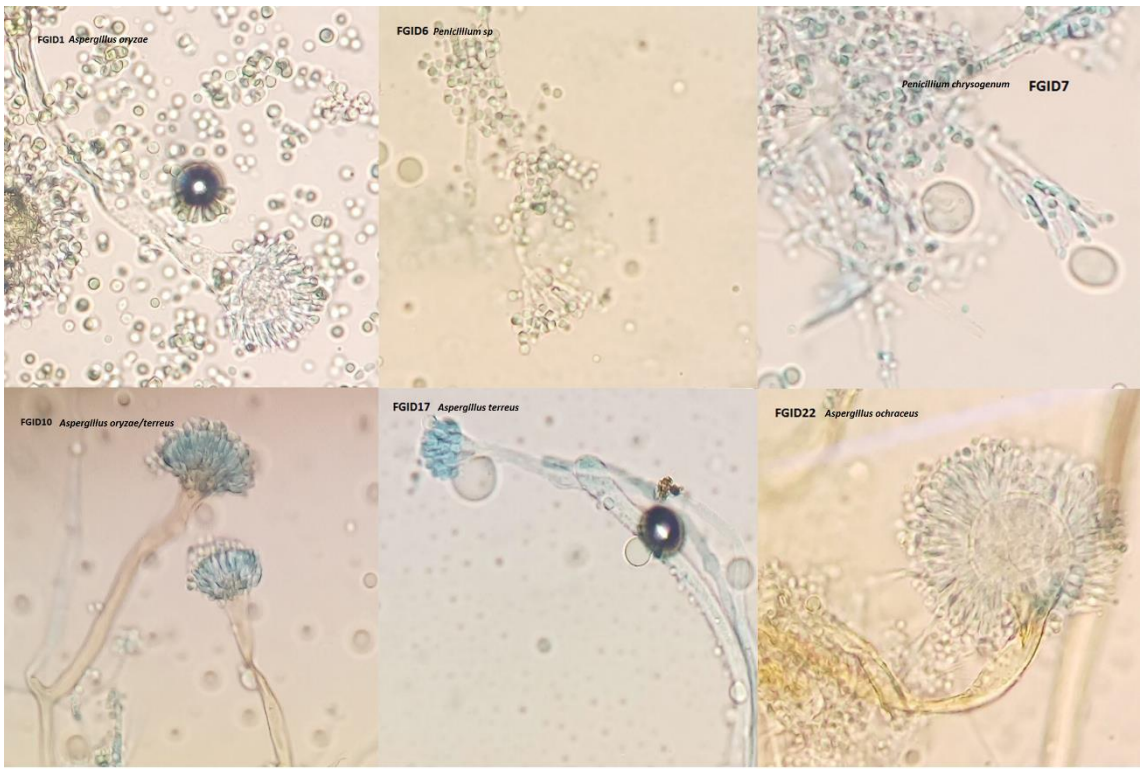
Annex Figure 2. Gram-positive cocci

C) Gram-Negative rods



Annex Figure 3. Gram-negative rods

D) Fungi



Annex Figure 4. Fungi

II. Risk-analysis criteria for determination of microbial monitoring points in aseptic zone qualification (Reig Jofre 2017)

A risk analysis was carried out in order to assess the number of sampling points for microbial monitoring during the qualification of a manufacturing aseptic zone. This annex explains the criteria to assign a risk factor to each of the activities carried out in the aseptic zone. Finally, the risk potential is calculated for every point taking into account the risk factors explained below.

The risk factor describes how critical for the product the risk would be and it is assigned as following:

- Very critical: 4
- Critical: 3
- Slightly critical: 2
- Little critical: 1

Risk factors can be divided into those due to installation, manufacturing process and products. Risks derived from installations include air recirculation and refluxes, among others. Risk associated to the manufacturing process depends on the classification of the room where the activity is taking place, the type of operation and the number of people present in the room. Finally, the risk of the product is always severe if the product has been exposed to the environment.

Factors taken into account in the risk analysis were the following:

Annex Table 1. Risk analysis criteria

Risk	Weighing	Description
Installation/Facilities		
Overpressures		
Yes	1	Little influence on product
No	2	Large influence on product
Room height		
Greater than 2.5 m	2	Large influence on product
Smaller than 2.5 m	1	Little influence on product
Air renewals		
25	2	Large influence on product
>25	1	Little influence on product
Personnel flow		
Close to the product	2	Large influence on product
Far from the product	1	Little influence on product
Air return		
Yes	2	Large influence on product
No	1	Little influence on product
Process		
GMP Classification		
A	4	High criticality
B	3	Medium-high criticality
C	2	Medium criticality
D	1	Little criticality
Operation		
Filling	4	Very large influence on product
Capping	3	Large influence on product
Manufacturing	2	Medium-large influence on product
Ancillary rooms	1	Little influence on product
Number of people		
>2	2	Large influence on product
2	1	Little influence on product
Product		
Product exposure		
Yes	2	High influence in results
No	1	Little influence in results