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# Rapid microbiological methods. They are rapid! Are they fast?

***João Henriques[1]; Catarina Cardoso[2]; Carla Vitorino[3,4]****

*[1]Faculty of Pharmacy, University of Coimbra, Portugal*

*[2]Laboratórios Basi, Parque Industrial Manuel Lourenço Ferreira, Portugal*

*[3]Center for Neurosciences and Cell Biology (CNC), University of Coimbra, Portugal*

*[4]REQUIMTE/LAQV, Group of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra, Portugal*

**Corresponding Author: Carla Vitorino**

Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

Email: csvitorino@ff.uc.pt

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**Abstract**

Rapid Microbiological Methods (RMM) are relatively recent methods of microbiology. When compared to traditional microbiological methods, that may take days or even weeks to deliver a result, RMM's output is given in a timely manner with increased precision and sensitivity. In an industrial environment, this could lead to numerous advantages such as early detection of microbiological contamination and increased control over the manufacture process. For example, RMM are a hot topic in pharmaceutical industry as the control of microbiological contamination still relies on a sterility test on finished product, over a period of 14 days. However, the costs associated with RMM implementation, validation difficulties and technical complexity has hampered these method's fast development and widespread use. This review provides an overview of the recent advances of the RMM, their applications in the pharmaceutical and food industries and the reasons for a not so fast implementation.

**Keywords:** Food industry; Implementation; Process analytical technology; Pharmaceutical industry; Rapid microbiological methods

**Abbreviations:** 6CFDA: 6-Carboxyfluorescein Diacelate; AOAC: Association of Analytical Communities; ATP: Adenosine Triphosphate; CFU: Colony Forming Units; DAPI: 4,6-Diamino-2-Phenylindole; DEFT: Direct Epifluorescent Filtration Technique; DNA: Deoxyribonucleic Acid; ELISA: Enzyme-Linked Immunosorbent Assay FDA: Food and Drug Administration; FISH: Fluorescent *in Situ* Hybridization; FTIR: Fourier-Transform Infrared IMC: Isothermal Micro calorimetry; IMS: Immuno-Magnetic Separation; MALDI-TOF: Matrix Assisted Laser Desorption/Ionization Time-of-Flight; PAT: Process Analytical Technology PCR: Polymerase Chain Reaction; Ph. Eur.: European Pharmacopoeia; RMM: Rapid Microbiological Methods; USP: United States Pharmacopeia

## Introduction

Microbiology and microbiological methods are transversal to many fields. Methods to assess the presence of bacteria, yeasts and molds, to quantify microorganisms or to identify the species present in a sample are routine procedures on several industries, such as pharmaceutical, biopharmaceutical, cosmetic, food, water industries. Historically, the referred analyses were done by using labor-intensive, Pasteurian, culture-based methods. However, these methods are not very sensitive (due to non-culturable microorganisms) and take a long time to deliver a result. The most iconic case is the sterility test performed on finished sterile drug products, which takes 14 days to deliver a result [1,2]. The growing need for more rapid results led to the development of the called Rapid Microbiological Methods (RMM). Some RMM, however, are considered rapid solely when compared to the culture-based methods. RMM result delivery strongly depends on the application and objective, varying from real-time to results usually delivered in up to 48 h. Most industries soon embraced and helped developing RMM. Pharmaceutical industry, however, for regulatory reasons and deployment issues was not so eager in implementing RMM in their manufacturing processes and product control [3]. This review provides an overview over the most common RMM, their applications and advancements and tries to understand why these rapid methods are not so fast when it comes to implementation, particularly in the pharmaceutical industry.

## Rapid microbiological methods

RMM are a broad group of analytical microbiological methods that share the common characteristic of delivering a result in less time than compendial, Pasteurian, labor-intensive methods.

Apart from the reduced time consumption advantage, RMM present other advantages over compendial methods, including the possibility of automation and increased sensitivity and accuracy [3,4].

The RMM may be classified under two different perspectives, namely, the type of determination (directly linked with the objective of the test) and the detection principle [1]. The type of determination of an RMM may be (i) qualitative, (ii) quantitative, or (iii) identification test. Qualitative RMM evaluate the presence or absence of viable bacteria, while quantitative RMM allows to enumerate microorganisms in a sample. Identification tests permit classification of a determined taxonomic group. However, these tests usually require a prior colony purification step.

The three basic detection methods are: (i) detection of growth, (ii) direct analysis, and (iii) analysis of cell components. The detection of growth is based on the detection of metabolic by-products (e.g. $CO_2$) or heat production from catabolic activity. Direct analysis uses the whole bacteria for detection, while analysis of cell components uses only parts of the cell for detection, such as Adenosine Triphosphate (ATP), nucleic acids or fatty acids. It is important to recognize that some methods may require a growth (i.e. enrichment) step for the signal to be measurable but do not use indicators of growth (e.g. metabolic by-products) as means of detection. Therefore, here these methods are not considered growth-methods. Table 1 provides a general overview of the existing RMM, their principle, the type of analysis the RMM is used for and information regarding sensitivity and time to deliver a result.

**Table 1:** General overview of the rapid microbiological methods.

| RMM | Type of determination | Detection method | Detection principle | Time to result | Ref |
|---|---|---|---|---|---|
| ATP bioluminescence | Qualitative; Quantitative | Direct analysis | Detection of bioluminescence of the reaction of ATP, present on living cells, with luciferase. | 1 h<br>4 to 7 days for sterility test | [5, 6] |
| Autofluorescence | Quantitative | Direct analysis | Detects fluorescence after illumination of filtered colonies with blue light. May require incubation to obtain colonies with desirable size. | 3 h for detection | [7] |
| Biochemical assays | Identification | Direct analysis | Detects the reaction of bacteria to certain biochemical substances (e.g. sugars). It is required a strain purification step. | According to the test, the microorganism and the strain purification step. | [1] |
| $CO_2$ detection | Qualitative | Detection of growth | $CO_2$ production (detected by a colorimetric reaction) is indicative of microbial growth. | 24-72 h for sterility test | [8] |
| DEFT | Qualitative; Quantitative | Direct analysis | Uses a double staining process with 6CFDA and DAPI to distinguish viable from non-viable cells. | < 1 h | [9] |
| Direct laser scanning | Quantitative | Direct analysis | Scanning of the filter surface after filtration with a laser | Few days | [1] |
| Fatty acid profile | Identification | Cell components | Makes use of a very stable fatty acid composition along the taxonomic group comparing the analyzed with a database. Requires a strain purification step. | 24-48 h | [10] |
| Flow cytometry | Qualitative Quantitative | Direct analysis | Detection of fluorophore-marked bacteria on a flow cytometer. | Few minutes | [11] |
| FTIR | Identification | Direct analysis | Uses the absorption of radiation on the infrared region of the whole bacteria. Requires that all the bacteria are on the same life stage. | 6-8 h (without culture step) | [12] |

| | | | | | |
|---|---|---|---|---|---|
| Genotypic methods | Qualitative; Quantitative; Identification | Cell components | PCR: Amplification of specific or universal highly conserved regions of nucleic acid. | < 2 h | [1, 13] |
| | | | Restriction endonuclease analysis: Mapping of the genetic profile by using restriction enzymes and comparing the fragments with a database. Requires a pure colony. | Few hours | |
| Headspace pressure | Qualitative | Detection of growth | Gas production or consumption by bacteria alters gas composition and pressure on a closed media vial. | 72 h | [14] |
| Immunological methods | Identification | Cell components | ELISA: Specific antibodies fixed to a well determine the binding of the of bacteria. Detection is made by a marked secondary antibody. | According to the detection method. | [18] |
| | | | IMS: Separation of bacteria by using specific antibodies marked with paramagnetic particles. Application of a moving magnetic field allows to separate the bacteria of interest. A coupled detector is needed. | 14 to 24 h for sterility test | |
| Mass spectrometry | Identification | Direct analysis Cell components | Compares the profile of ions (by relative abundance and mass to charge ratio) to a database. | < 40 min | [19] |
| Microcalorimetry | Qualitative | Detection of growth | Measurement of heat released from microbial catabolic activity. | 24-72 h | [20] |
| Phage-based methods | Qualitative; Identification | Direct analysis Cell components | Detection of protein expression or intracellular material after phage infection. As phage may be specific to a determined host, the method may be used for identification. | 48 h | [21] |
| Solid phase cytometry | Qualitative; Quantitative | Direct analysis | Detection of bacteria after filtration and marked with an intracellularly converted fluorophore. | 3 h for sterility test | [22] |
| Turbidimetry | Qualitative | Direct analysis | The presence of bacteria (either viable or non-viable) modifies the medium optical density. | According to the growth time. | [1] |

**Note:** 6CFDA: 6-carboxyfluorescein diacelate; ATP: Adenosine triphosphate; CFU: Colony forming units; DAPI: 4,6-diamino-2-phenylindole; DEFT: Direct epifluorescent filtration technique; DNA: Deoxyribonucleic acid; ELISA: Enzyme-Linked Immunosorbent Assay; FTIR: Fourier-transform infrared; IMS: Immuno-magnetic separation; PCR: Polymerase chain reaction; RMM: Rapid microbiological methods.

## Application

As previously mentioned, RMM crucially reduces time to result. This feature is of great importance in an industrial environment because the rapid availability of results allows faster release of batches, to take corrective actions, if necessary, earlier batch rejection, improved manufacturing consistency and consequent waste reduction. The sensitivity improvement also enables greater microbiological product quality and control. Under this perspective, RMM implementation is transversal to all the industries that make use of microbiology, such as the pharmaceutical, biopharmaceutical, cosmetics, water and food industries.

### Pharmaceutical industry

In the pharmaceutical industry, RMM implementation can bring significant advantages as microbiological control, either as in-process control of bioburden or as final product control, since this is part of the specifications of batch release of many drug products. Sterile drug products, for instance, are controlled by the end-product sterility test, described on general chapters 2.6.1 of the European Pharmacopoeia (Ph. Eur.) [1] and <71> of the United States Pharmacopeia (USP) [2]. Sterility test is carried out using the membrane filtration technique (only for filterable drug products) or by the direct inoculation technique. According to the membrane filtration technique, the product is filtered using membranes with nominal pore size ≤ 0.45 µm and 50 mm of diameter. After filtration, the membrane as a whole or cut into two halves is transferred to appropriate growth media (i.e. fluid thioglycollate medium, soya-bean casein digest medium or another appropriate medium commercially available). In the direct inoculation technique, the growth media is directly inoculated with the drug product and the microbial growth (e.g. media turbidity) is then observed. The sterility test takes 14 days to deliver a result and has several other problems: (i) statistical limitations regarding the sampling process, (ii) non-cultivable microorganisms may be present and not detected, (iii) the quarantined units of the batch occupy storage space for a long time, (iv) microorganisms may be under stress from the manufacture process conditions and may not be detected [23,24]. RMM employment, with their increased sensitivity and reduced time to result, would then translate into more safety for the patient, because the product would be more tightly and in a timely manner controlled. Production costs would be lower, due to reduced laboratorial testing and faster batch release [3]. Lower production costs would therefore result in decrease of patient and healthcare services expenses.

The areas that may be covered with RMM implementation in the pharmaceutical industry are environmental monitoring on clean rooms, microbiological control of raw materials (including water testing), finished non-sterile product testing, sterility test [4] and antimicrobial effectiveness tests [25]. RMM applications regarding environmental monitoring consist of air monitoring [26-28], isolator integrity [29] and surfaces monitoring [30].

Bioburden control and non-sterile products microbiological

control require a 2 to 7 days growth period, while the sterility test requires the samples to be incubated for 14 days. However, over the last years, a growth of commercial setups that claim to be suitable to substitute the compendial sterility test has been noticed (*vide* Table 2).

**Table 2:** Commercial apparatuses for sterility testing and claimed time for detection.

| Apparatus | Company | Principle | Time to result | Ref |
|---|---|---|---|---|
| BacT/Alert® 3D Dual-T | BioMérieux® | $CO_2$ detection | 24-72 h | [31] |
| BacTrac® 4300 | Sy-Lab® | Impedance | 14-24 h | [18] |
| Celsis Accel® System | Charles River® | ATP luminescence | 4-7 days | [6] |
| Milliflex® Rapid System | Merck® | ATP luminescence | 5 days | [32] |
| ScanRDI® | BioMérieux® | Solid-phase cytometry | 3 h | [33] |

**Note:** ATP: Adenosine triphosphate.

Parveen *et al*. [8] evaluated the Milliflex® Rapid System, the BacT/Alert® and the BACTEC® systems as surrogates to the compendial sterility test on vaccines. For that, the team prepared inocula of 14 microorganisms in aliquots of 10 mL of two different matrices (inactivated influenza vaccine and a fluid with 0.1 % of peptone) containing 0.1, 1, 10 and 100 Colony Forming Units (CFU). These aliquots were used to inoculate the different media for each test. The Milliflex® Rapid System revealed significantly higher sensitivity ($p<0.05$) at low microorganism concentrations (i.e. 1 and 0.1 CFU/10 mL) than compendial methods, whereas the BacT/Alert® and the BACTEC® systems showed similar and significantly less sensitivities than compendial methods ($p<0.05$) for concentrations of 0.1 CFU/10 mL and 1 CFU/10 mL, respectively. The time for detection of all microorganisms by the Milliflex® Rapid System was within 5 days, being in accordance to the manufacturer claims [32]. For most microorganisms, the BacT/Alert® and the BACTEC® systems showed the quicker time to detection (within 72h). However, for slow growing microorganisms (e.g. *Propionibacterium acnes*), there was no significant difference of time to detection when compared to the compendial methods (around 11 days). The authors acknowledge the potential of Milliflex® Rapid System for substitution of the compendial sterility testing. The BacT/Alert® and the BACTEC® systems arise as good alternatives for products without preservatives that can only be tested by direct inoculation.

The comparison of compendial sterility testing with commercial apparatuses was also done by other authors. Smith and co-workers [22] evaluated SCANRDI® with 8 microorganisms and demonstrated statistically non-inferiority of limit of detection when compared to the pharmacopoeial sterility test. Kaiser and collaborators [34] compared the BACT/ALERT® 3D Dual-T with the direct inoculation test. The commercial method complied with all validation requirements and the possibility of automation reduced the probability of occurrence of human error. Bugno et al. compared the sterility testing with BACT/ALERT® 3D Dual-T on spiked commercial 100 mL bags of 0.9 % of NaCl [35] and Celsis AKuScreen™ Advance™ system on spiked batches of 0.9 % of NaCl, 5 % dextrose, Ringer solution with lactate and metronidazole [36]. Both revealed equivalent performance compared to pharmacopoeial methods and more rapid detection.

According to the way the RMM are implemented they can be considered as Process Analytical Technology (PAT) [28,37,38]. In fact, the first PAT application approved by the Food and Drug Administration (FDA) after the publication of the guidance "PAT-A Framework for Innovative Pharmaceutical Development, Manufacturing and Quality Assurance" [39] was the implementation of RMM for microbial-limit testing of water and release of some dosage forms, by Glaxo Smith Kline [40].

### Implementation constraints

The implementation of RMM by the pharmaceutical industry has been, ironically, slow. The reasons for this fact are two-folded: at the method level and at the regulatory-industrial level. On the one hand, the implementation of an RMM implies costs on instrumentation and validation, which may not have return in a timely manner. On the other hand, because of the high level of regulation, pharmaceutical industry has the tendency to be precautious in innovation implementation. From the industrial perspective, the probability of disapproval by the regulatory authorities means loss of time and resources. For existing products, the modifications of the dossier by implementation of a new analytical method, such as an RMM, also imply costs.

RMM validation for the pharmaceutical industry is addressed on general chapter 5.1.6 "Alternative Methods for Control of Microbiological Quality" of Ph. Eur. [1] and on general chapter <1223> "Validation of Alternative Microbiological Methods" of USP [2]. On these chapters the principles of validation of RMM for European Union and United States, respectively, are described. Table 3 provides a comparative overview of the requirements of each Pharmacopoeia. It is noteworthy that the USP does not address identification tests on the respective general chapter.

**Table 3:** Comparative overview of the validation requirements for RMM on the European Pharmacopoeia and the United States Pharmacopeia.

| Parameter | Qualitative Tests | | Quantitative Tests | | Identification Tests |
|---|---|---|---|---|---|
| | Ph. Eur | USP | Ph. Eur | USP | Ph. Eur |
| Accuracy | X | | X | X | X |
| Precision | X | | X | X | X |
| Specificity | X | X | X | X | |
| Limit of Detection | X | X | | X | |
| Limit of Quantification | | | X | X | |
| Linearity | | | X | X | |
| Operational Range | | | X | X | |
| Robustness | X | X | X | X | X |
| Repeatability | | X | | X | |
| Ruggedness | | X | | X | |
| Equivalency | | X | | X | |

**Note:** Ph. Eur: European Pharmacopeia; USP: United States Pharmacopeia; X: Required.

After the enumeration of the parameters to be addressed on validation of RMM, the general chapter 5.1.6 of the Ph. Eur. [1] proceeds referring the principles of primary validation (by the supplier), validation for the intended use (by the user), i.e. verification of the suitability of the RMM to the intended situation, and specific guidance for some RMM. Finally, a step by step example of validation is provided.

USP general chapter <1223> [2] introduces four alternatives to the demonstration of equivalence of RMM: (i) acceptable procedures, where a standard inoculum of a specific microorganism is used, (ii) equivalence of performance, where validation parameters are compared with the compendial methods, (iii) equivalence of results, that deals with comparison of numerical results or correlation with results obtained by compendial methods, and (iv) decision of equivalence, for qualitative methods, where the result absence or presence of bacteria is compared.

The inclusion of pharmacopoeial general chapters regarding validation of RMM, with a step-by-step example of validation shows a willingness to accept RMM as surrogate to the compendial microbiological methods. This will to accept RMM may be seen in already approved examples [40,41]. However, the differences between the validation requirements of the pharmacopoeias (Table 3) pose a problem to a company with businesses across different regions. This problem may be easily overcome by a process of pharmacopoeial harmonization. Finally, RMM implementation is hindered by the necessary volume of investment. If the investment on RMM equipment, consumables and implementation is not recovered in a timely manner, RMM will not be seen as a viable alternative nor will be widely implemented.

### Food industry

Food industry has been more eager than pharmaceutical industry in the implementation of RMM and has been one of the driving forces for development of new RMM [42]. In fact, there are reports of application of RMM to food matrices since the 70's [43,44]. This may be due to the lower sensitivity needs in the food industry when compared to absolute sterility need of parenteral drug products. However, it is noteworthy that the complexity and great variety of food samples often poses great difficulties to method validation. Validation of RMM for the food industry is usually based on guidelines of the Association of Analytical Communities (AOAC), for the United States of America and on ISO 16140 for the European Union [45].

RMM found place on the food industry for monitoring of the microbiological quality of food products, for facilities hygiene monitoring and for enumerating the food-producing microorganisms. The monitoring of the microbiological quality of food products is important to reduce the probability of foodborne infections. Some examples include ATP bioluminescence for determination of microbial load on poultry [46], mango surface [47] and soy milk [48]; flow cytometry for bacteria enumeration on cow milk [49] and for *Escherichia coli* and *Shigella Spp*. detection [50]; Polymerase Chain Reaction (PCR) for identification of *Listeria monocytogenes* [51]; Immunomagnetic Separation (IMS) coupled with an impedance sensor for rapid detection of *Salmonella Typhimurium* and *Escherichia coli* O157:H7 [52] and Matrix Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectroscopy for rapid identification of *Listeria monocytogenes* in dairy products [53]. The hygiene monitoring has been assured primarily by ATP bioluminescence [54-56]. Other techniques include Direct Epifluorescent Filtration Technique (DEFT) and direct epifluorescent microscopy [57]. The enumeration of food-producing microorganisms is useful for assessment of bacterial populations during food processing. This issue has been addressed by turbidimetry [58], flow microcalorimetry [59], flow cytometry [11,60] and Fluorescent *In Situ* Hybridization (FISH) [61].

### Recent developments

This section is intended as a repository of some of the most recent RMM developments, spanning different methods and applications, while revealing the RMM large applicability range.

Brueckner and co-workers [20] assessed the possibility of using Isothermal Microcalorimetry (IMC) as surrogate of the sterility test. Finished product samples were inoculated with 0.1 mL of a microorganism (7 different microorganisms) suspension containing <10 CFU/0.1 mL. The final volume was between 12 and 16 mL. The comparison method was the visual inspection of growth. In all samples, the growth was detected and in 6 out of 7 microorganisms, the detection time was significantly lower ($p<0.01$) for IMC. The authors recognized the potential of IMC to substitute the pharmacopoeial sterility test.

Sorensen *et al*. [62] studied the applicability of online fluorescence spectroscopy to assess the microbial quality of untreated drinking water in real-time. Four aquifers with history of contamination during heavy rainfall in England were chosen. They used an excitation wavelength of 285 nm and detection at 365 nm and 450 nm (tryptophan-like fluorescence and humic like fluorescence, respectively). Regular samples in duplicate (one for *Escherichia coli* analysis and another one for fluorescence, absorbance and total bacterial count by flow cytometry) were taken to compare with online analysis results. The correlation (Spearman's rank correlation coefficient) of tryptophan-like fluorescence and humic-like fluorescence with *Escherichia coli* count was, respectively, 0.71 ($p<0.001$, n=134) and 0.77 ($p<0.001$, n=122), whereas the turbidity (usual method to real-time assessment of water quality) correlation is 0.48 ($p<0.001$, n=134). The correlation of tryptophan-like fluorescence and humic-like fluorescence with total bacterial cell count was similar to the correlation with *Escherichia coli* count being, respectively, 0.73 ($p<0.001$, n=124) and 0.76 ($p<0.001$, n=119), while the turbidity correlation was 0.40 ($p<0.001$, n=124). Therefore, the authors concluded that the online fluorescence is an effective method to monitor the microbial quality of water. However, the turbidimetry analysis should not be discarded as it gives precious information regarding suspended solids on water.

Saulnier and co-workers [13] developed and validated a method to rapidly identify all *Vibrio splendidus*-related strains usually found in some cultured oysters (*Crassostrea gigas*) using Duplex Taqman real-time PCR. The team selected the sequence 16S rRNA, present in almost all *Vibrio splendidus*-related strains but not in other *Vibrio*. Of the 44 *Vibrio* species, 14 produced positive results. Of these 14, all were *Vibrio splendidus*-related strains except for the *Vibrio aestuarianus*. Only the *Vibrio splendidus*-related strains without the sequence 16S rRNA yielded negative results. The team found that the specificity of the method to the *Vibrio splendidus*-related strains and *Vibrio aestuarianus* was 100 %.

A technique based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used by Thouvenot and collaborators [19] to rapidly (< 40 min) identify the

different *Listeria* species from isolates. The validation was made with 386 isolates, representative of the diversity of the *Listeria* genus. The negative control was a set of 34 isolates with species frequently misidentified as *Listeria*. The results were compared with conventional identification tests, i.e. API-*Listeria* and haemolysis test. All the samples were correctly identified and none of the negative controls was misidentified as *Listeria*. When applying the method to prospective identification (n = 1201), the isolates were all correctly identified.

Pane *et al*. [11] applied a flow cytometry method to the quantification of *Lactobacillus rhamnosus* GG on a probiotic finished product. The method was validated by assessing its accuracy, precision, ruggedness, linearity, limit of quantification, specificity, robustness and range. The method suited the application.

*Escherichia coli* is an indicator of water quality and the O157:H7 strain, for its pathogenicity, is of particular interest because it is easily detected. For this purpose, Ngamsom *et al*. [63] coupled immunomagnetic separation *via* immiscible filtration assisted by surface tension to ATP bioluminescence to rapidly (20 min) quantify *Escherichia coli* O157:H7 in a sample. In a microfluidic chamber, two immiscible liquids create a virtual wall. Bacteria on the sample chamber are captured by specific antibodies functionalized with a superparamagnetic particle. By applying a moving magnetic field, the active (i.e. connected to bacteria) antibodies are transferred through chambers to the detection chamber, where bacteria are detected by ATP bioluminescence. The system is able to detect concentrations of 6 CFU/mL. The method was tested with *Escherichia coli* O157:H7 spiked wastewater effluents and enabled detection of concentrations of $10^4$ CFU/mL.

Santangelo and collaborators [64] developed a 3D printed microfluidic chip that allows real-time (4 s response and 17 s to recover to baseline) and sensitive ATP detection. The system, which is low-cost, disposable, miniaturized and reduces reagent consuming, is coupled to a silicone photomultiplier tube detector. Seven ATP solutions with concentrations ranging from 15 nM to 1 µM were used to compare the performance of the microfluidic chip with a commercial bioluminometer. The error was below 3 % and the error between repetitions was up to 20 %. The limit of determination was 8 nM of ATP. The system was tested with a solution of a lysate of *Escherichia coli* O157:H7 (made from a suspension with cell density of $10^8$ CFU/mL) and results showed that performance was not affected. This chip provides the capability of direct measurement inside environmental chambers.

## Conclusion

RMM has seen a great development for the most diverse usages, and with transversal application to the various fields where microbiology is needed. At the industrial level, RMM are appealing due to advantages that are offered, particularly, the better assurance of quality and the reduced time of analysis.

Several industries have already keenly adopted RMM into their manufacturing processes and controls. The exception to this rule is the pharmaceutical industry. Although there are some cases of success [40,41], RMM implementation is still slow. The reasons are of two interconnected orders (i.e. method level and regulatory-industrial level). These reasons may be summarized in five points:

(i) implementation and validation costs,

(ii) need for very low limits of detection when compared to other industries (the need to detect 1 viable cell usually leads to a great increase of time to result),

(iii) possibility of disapproval by regulatory authorities,

(iv) costs associated with marketing authorization post-approval changes, and

(v) discrepancies across pharmacopoeias.

These reasons have been hindering RMM wide implementation. However, the recent technological progress in the area suggests that more low cost, robust, sensitive and rapid technologies will appear. In the meanwhile, as incentive for RMM implementation, a process of pharmacopoeial harmonization gathering the general chapters 5.1.6 of Ph. Eur. and <1223> of USP is highly encouraged, in order to overcome discrepancies of validation requirements across different regions.

RMM are in fact rapid, when compared to compendial methods. Are they fast? There is still a lot of work to do.

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