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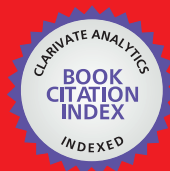
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# **Use of ATP Bioluminescence for Rapid Detection and Enumeration of Contaminants: The Milliflex Rapid Microbiology Detection and Enumeration System**

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## **1. Introduction**

Rapid microbial detection becomes increasingly essential to many companies in pharmaceutical, clinical and in food and beverage areas. Faster microbiological methods are required to contribute to a better control of raw materials as well as finished products. Rapid microbiological methods can also provide a better reactivity throughout the manufacturing process. Implementing rapid technologies would allow companies for cost saving and would speed up products release. Despite clear advantages, traditional methods are still widely used. Current methods require incubation of products in liquid or solid culture media for routinely 2 to 7 days before getting the contamination result. This necessary long incubation time is mainly due to the fact that stressed microorganisms found in complex matrices require several days to grow to visible colonies to be detected. Moreover, this incubation period can be increased up to 14 days in specific application like sterility testing for the release of pharmaceutical compounds. Although these techniques show advantages like simplicity, the use of inexpensive materials and their acceptability to the regulatory authorities, the major drawback is the length of time taken to get microbiological results. Thus, face to the growing demand for rapid detection methods, various alternative technologies have been developed. In the field of rapid microorganisms detection, ATP-bioluminescence based on luciferine/luciferase reaction has shown great interest. Indeed, adenosine triphosphate (ATP) is found in all living organisms and is an excellent marker for viability and cellular contamination. Detection of ATP through ATP-luminescence technology is therefore a method of choice to replace traditional method and significantly shorten time to detection without losing reliability.

This chapter will address the ATP-bioluminescence principle as a sensitive and rapid detection technology in the Milliflex® Rapid Microbiology Detection and Enumeration System (RMDS). This system combines membrane filtration principle, detection of microorganisms by ATP-bioluminescence and light capture triggered by a Charged Coupled Device camera (CCD) followed by software analysis.

## 2. ATP-Bioluminescence

### 2.1 ATP-Bioluminescence principle

Light-producing living organisms are widespread in nature and from diverse origins. The process of light emission from organisms is called bioluminescence and represents a chemical conversion of energy into light. Since the work of William D McElroy showing that ATP is a limiting and key factor of the bioluminescent reaction, research has lead to a better understanding of how light is produced by fireflies (McElroy, 1947; McElroy, 1951; McElroy et al., 1953). The bioluminescence mechanism involving Luciferase enzyme is a multistep process which mainly requires Luciferin substrat, Oxygen (O<sub>2</sub>), Magnesium cation (Mg<sup>++</sup>) and ATP (DeLuca & McElroy, 1974; McElroy et al., 1953; Seliger, 1989). ATP-bioluminescence using luciferine/luciferase relies on luciferine oxidation by the luciferase and the integrated light intensity is directly proportional to ATP contents. Luciferase converts in presence of ATP and Magnesium firefly D-luciferin into the corresponding enzyme-bound luciferil adenylate. The luciferil adenylate complex is then the substrate of the subsequent oxidative reaction leading to oxyluciferin. The light emission is a consequence of a rapid loss of energy of the oxyluciferine molecule from an excited state to a stable one. This reaction induces the emission of photons with a efficient quantum yield of about 90% (Seliger, 1989; Wilson & Hasting, 1998) (Fig1).

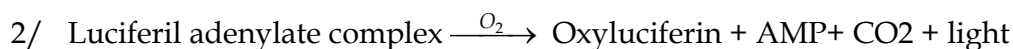
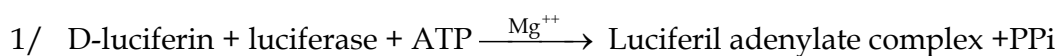


Fig. 1. Chemical reactions of the ATP-bioluminescence based on luciferin/luciferase system (PPi:inorganic pyrophosphate, CO<sub>2</sub>: Carbon Dioxide). Photons of yellow-green light (550 to 570 nm) are emitted.

### 2.2 Luciferase protein

Luciferase is a common term used to describe enzymes able to catalyze light emission. Luciferase belongs to the adelynate-forming protein family and is an oxygen-4-oxidoreductase gathering decarboxylation and ATP-hydrolysing main activities. Structural studies have shown that *Photinus pyralis* Luciferase protein is folded into 2 domains: a large N-terminal body and a small C-terminal domain linked by a flexible peptide creating a wide cleft (Conti et al., 1996). Amino acids critical for bioluminescence phenomenon belong mainly to the N-terminal domain (Branchini et al., 2000; Thompson et al., 1997; Zako et al., 2003). This implies that luciferine-binding site is mediated by conformational change to bring the 2 domains closer. This conformational change is consistent with the study of Nakatsu et al (2006) showing that luciferase from *luciola cruciata* exists in an “open form” and in a “closed form”, the later form creates an hydrophobic pocket around the active site and is responsible of light emission. Two kinds of colored light emission are described for luciferine/luciferase reaction. The typical high energy yellow-green light emission with a peak at 562 nm at pH 7.5 and red light emission with a peak at 620nm when the pH decreases to 5 (Seliger et al., 1964; Seliger & McElroy, 1964). This surprising phenomenon where Luciferase is able to emit light of different colors is not clearly understood but the isolation of colored luciferase variants shows that single amino acid substitution in

N-terminal domain affects bioluminescence color by modulating slightly the polarity of the active site environment (Hosseinkhani, 2011; Shapiro et al., 2005). This interesting feature opens the way to wide applications in biotechnology (Branchini et al., 2005).

### 2.3 ATP-Bioluminescence applications

With the isolation, cloning and purification of various luciferases from many bioluminescence-producing organisms (bacteria, beetles, marines organisms, etc), bioluminescent assays have been developed and widely used in microbiology to detect bacterial contamination by measuring presence of ATP and in molecular and cellular biology with luciferase as reporter gene to monitor gene expression, protein-protein interaction, etc (Francis et al., 2000; Roda et al., 2004; Thorne et al., 2010). The average intracellular ATP content in various microorganisms has been quantified and ATP has been shown to be a reliable biomarker of the presence of living organisms (Kodata et al., 1996; Thore et al., 1975; Venkateswaran et al., 2003). To be able to specifically detect living organisms by ATP-bioluminescence, the first step is to extract ATP from cells. This step is critical and impacts directly the reliability of the detection (Selan et al., 1992). Chemical solution or physical extraction methods were used in liquid samples (Selan et al., 1992; Siro et al., 1982). Some false negative results were described in few studies (Conn et al., 1975; Kolbeck et al., 1985). Additional studies investigated the cause of false negative results and demonstrated that ATP extraction was not efficient. Indeed, extensive sonication of bacterial samples for instance caused a significant increase of Relative Light Unit (RLU) measured (Selan et al., 1992). Taking into account this limitation, ATP-bioluminescent assay has already proved to provide good detection properties in many areas. Bioluminescent assay is broadly used to monitor air and surface cleanliness and product quality mainly in food industries and in less extent in pharmaceutical industries (Aycicek et al., 2006; Bautisda et al., 1995; Davidson et al., 1999; Dostalek & Branyik, 2005; Girotti et al., 1997; Hawronskyj & Holah, 1999). Studies shows that the level of contamination assessed though surface swabbing, ATP extraction and bioluminescent assay correlate well for 80 % of the samples tested with traditional plate method (Poulis et al., 1993). Availability of sensitive luminometers as well as many commercial ATP-bioluminescent kits has allowed the development of various protocols and applications in industrial microbiology. Currently, ATP- bioluminescence is an accepted and common technology used to monitor contamination in areas such as food and beverage, ecology, cosmetic, and clinical (Andreotti & Berthold, 1999; Chen & Godwin, 2006; Davidson et al., 1999; Deininger & Lee, 2001; Frundzhyan & Ugarova, 2007; Miller et al., 1992; Nielsen & Van Dellen, 1989; Selan et al., 1992; Yan et al., 2011).

## 3. Milliflex rapid microbiological detection and enumeration system

### 3.1 System description

RMDS offers a way to detect and quantify living microorganisms grown on a membrane. By combining ATP-bioluminescence and sensitive detection system, the microbial detection is obtained more rapidly than traditional method. In order to detect a colony or a micro-colony on a membrane by ATP-bioluminescence, the first step is to release ATP from cells. This critical step is achieved by nebulizing automatically an ATP-releasing solution onto the

membrane. ATP extraction is made on microcolonies grown on membrane which represents an advantage compared to chemical or physical extraction in liquid. Once ATP is released from lysed cells, it becomes accessible to bioluminescent reaction. A second solution is then automatically nebulized onto the same membrane. This solution brings to lysed cells all components, except ATP, involved in the Luciferin/Luciferase bioluminescence chemical reaction. A spray station is used to uniformly apply small volumes of reagents onto the membrane. As soon as bioluminescent reagents are sprayed onto the membrane, the bioluminescence reaction starts and photons are emitted. The membrane is then transferred manually from the spray station to the detection system. The Milliflex Rapid detection system combines the use of a highly sensitive CCD camera to monitor light emitted from microorganisms and an image analysis software to analyze the signal and give the number of microorganisms counted. The figure 2 shows the detection tower components and their function.

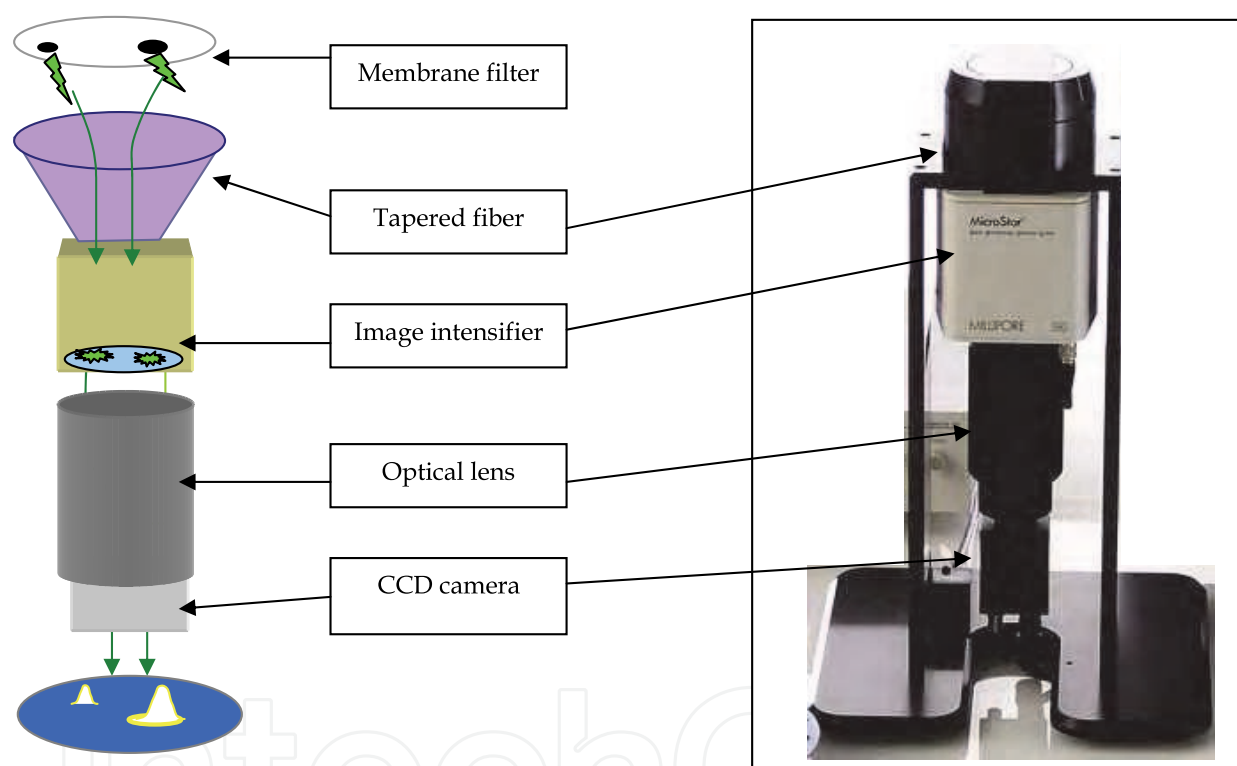


Fig. 2. Milliflex detection tower components: RMDS collects, amplifies, and registers on a CCD camera the light activity of bioluminescent reaction. Photons emitted by microorganisms go through the tapered fiber in order the light to be concentrated and becomes compatible with the size diameter of the CCD camera. In the intensifier, photons hit a photocathode and each photon is converted into cloud of electrons. Then electrons hit a phosphorous screen and are converted back into photons. The CCD camera records light every 30 times per second.

Data collected by the CCD camera are analyzed and treated by software to build an image of the membrane loaded on the top of the detection tower. The image indicates the place where light is emitted. As the signal is collected over a short period (integration time), spots size on the picture represents the light intensity accumulated or emitted by microorganisms (Fig.3).



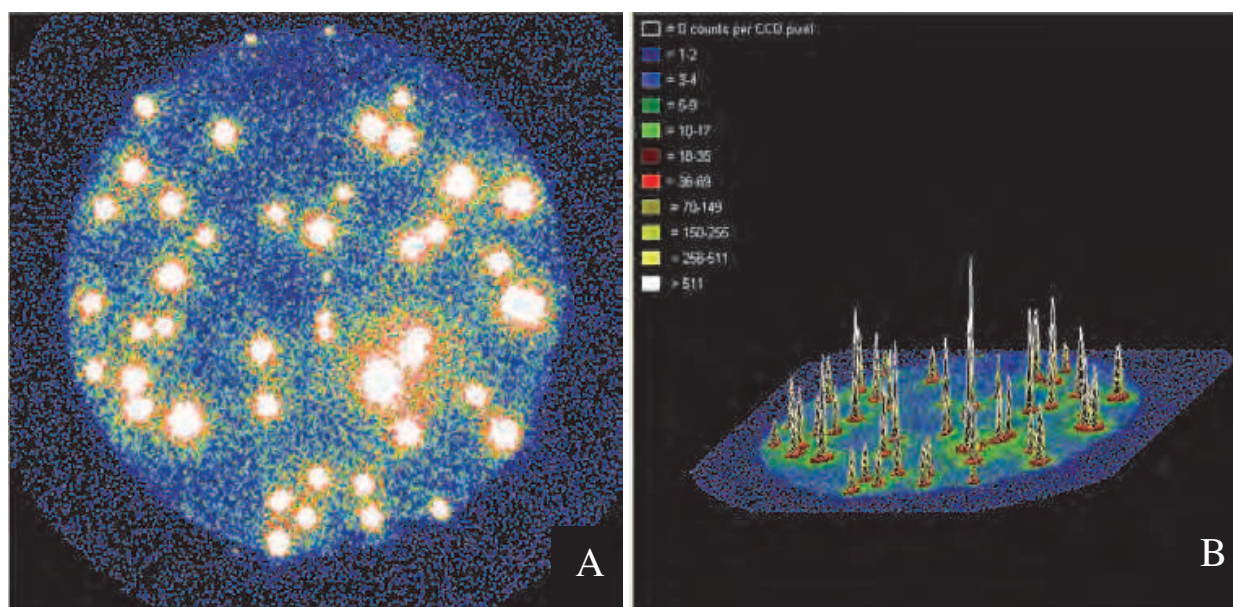


Fig. 3. Example of image given by RMDS software. Picture show the image of the membrane with spots (A) or peaks in 3 dimensions (B) representing exactly the place on the membrane where light is emitted. The result in colony forming unit is directly given by the system.

### 3.2 RMDS ATP-Bioluminescence protocol

The RMDS ATP-bioluminescence protocol includes the following steps:

1. filter the sample through a Milliflex funnel; 2. incubate the sample onto media; 3. separate the membrane from the media and let the membrane dry inside a laminar flow hood; 4. spray the ATP-releasing reagent and bioluminescence reagent onto the membrane by means of the Milliflex Rapid Autospray Station. The reaction between the ATP from microorganisms and the luciferase enzyme produces light; 5. place the membrane onto the detection tower and initiate detection and enumeration. Photons are detected by the system via a photon counting imaging tube coupled to a CCD camera. The photons generated by the ATP bioluminescence reaction are captured, and the integrated picture is displayed on the computer monitor; 6. after data treatment, a picture of the membrane is provided in two dimensions (2-D) exhibiting spots that represent colonies and in three dimensions (3-D) with peaks that correlate with the ATP content of the colony. The result is directly displayed in colony-forming unit (cfus) on the software screen. The successive steps are summarized in Fig. 4.

The standard protocol, performed in parallel, includes the following steps:

1. filter the sample through a Milliflex funnel; 2. incubate the sample and visually count cfus after incubation.

### 3.3 Evaluation of Luciferin/Luciferase relative concentrations for optimal detection of microorganisms

The relative concentrations of the 2 key components of the detection reagents were evaluated.

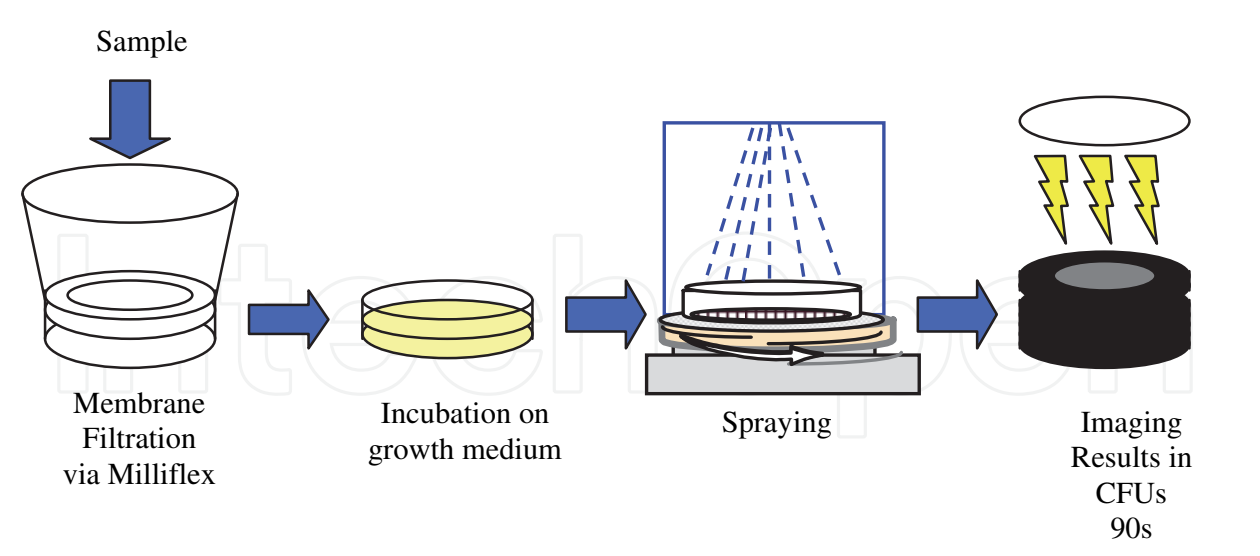


Fig. 4. RMDS ATP-bioluminescence protocol

The protocol used is described in the previous paragraph “RMDS ATP bioluminescence protocol”. Only the reagent used for detection varies for the 2 components relative concentrations as described in table 1.

	Formulation 1	Formulation 2	Formulation 3	Formulation 4	Formulation 5
Luciferase	3x	1.5x	1x	1.5x	1x
Luciferin	1x	1x	1x	0.5x	0.5x

Table 1. Formulations relative concentrations of Luciferin/Luciferase tested

The signal and background were determined using membranes incubated during 6h at 32.5°C on Tryptic Soy Agar inoculated with *Escherichia coli* or *Staphylococcus aureus* (table 2). Formulation 1 gave a signal so strong that the detection system was almost saturated. This saturation did not allow the accurate detection of bacteria on the membrane. The same issue occurred to a weaker extent using formulation 2. On the other hand, while the detection of *S. aureus* was accurate using formulation 5, the signal was too weak to allow all colonies of *E. coli* to be counted. Formulations 3 and 4 were both able to generate a good signal associated with low background. We can conclude from these results that the luciferin and luciferase concentration can be increased to optimize the signal but also that the balance between the 2 components is key. Signal will be increased while increasing concentrations but background as well. Formulation 3 which benefits from the best signal on background ratio has been used during the rest of the studies presented here. It is noticeable that depending on the application, the type of sample tested and the resulting background, this luciferase to luciferin balance can be adjusted to better match the detection criteria and increase signal on background ratio.

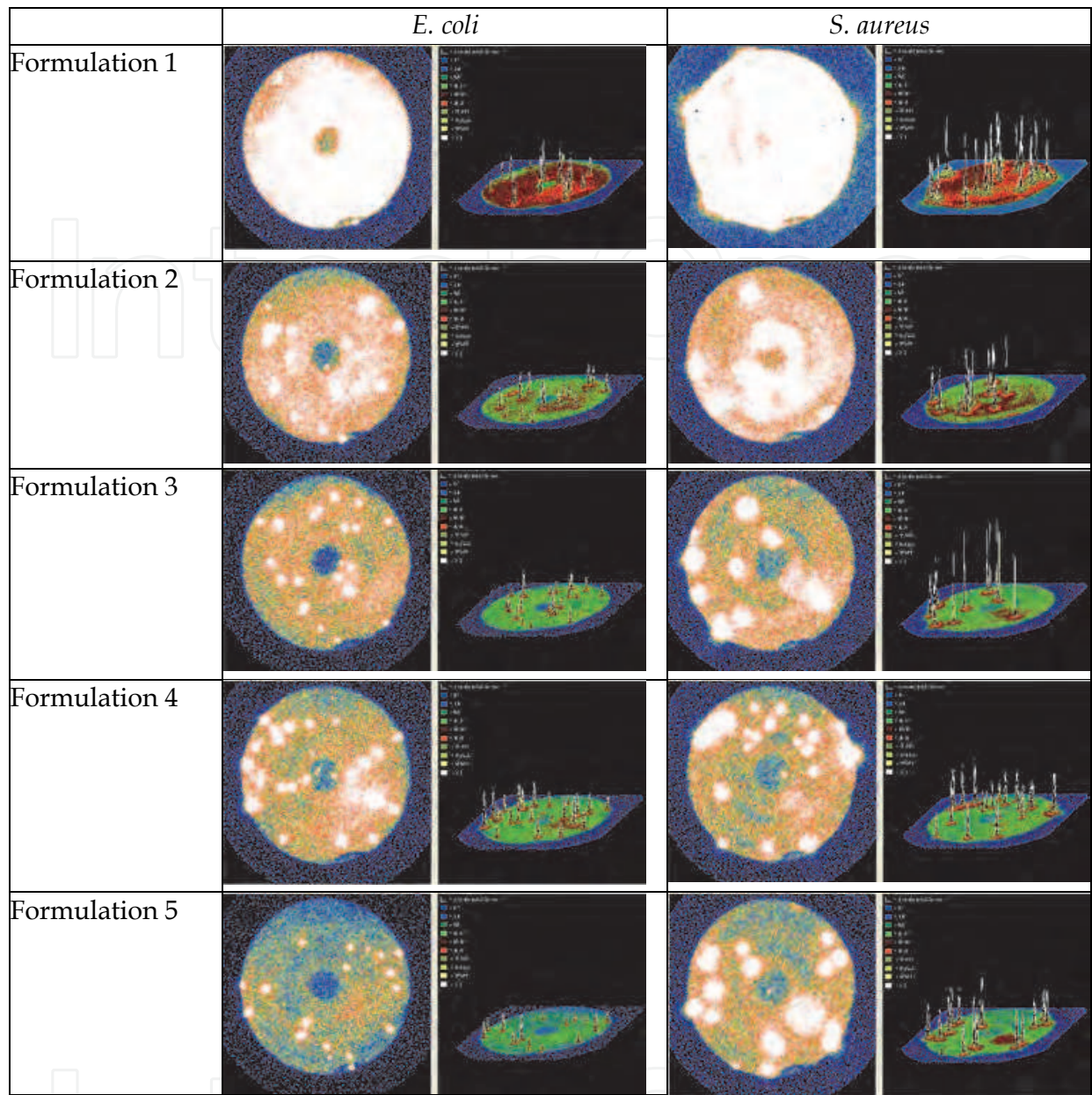


Table 2. RMDS results obtained with the 5 formulations of Luciferin/Luciferase tested

3.4 ATP background removal

One advantage to use an ATP bioluminescent assay to detect microorganisms is that ATP is present in all living organisms and is an excellent and sensitive biomarker of contamination. However this advantage can become an issue when non microbial or extracellular ATP is detected, generating bioluminescent background and preventing a reliable detection. Extracellular ATP is usually found either in culture media or in products containing eukaryotic cells. In both cases, the presence of unwanted ATP generates an overestimation of the contamination and impacts negatively the sensitivity of the ATP-bioluminescent assay. Two approaches are commonly used to remove extracellular ATP: enzymatic treatment to cleave ATP and lysis treatment to selectively lyse non bacterial cells. Methods including a treatment with ATP dephosphorylating enzymes such as apyrase or adenosine



phosphatase, have been described and used to remove efficiently ATP (Askgaard et al., 1995; Thore et al., 1975). Combination of apyrase and adenosine phosphate deaminase showed a good reduction of extracellular ATP and was applied to successfully detect *E. coli* and *S. aureus* in media broth and biological specimens (Sakakibara et al., 1997). When the objective of the assay is to detect and quantify bacterial contamination from a mixed population containing eukaryotic cells and bacteria, a differential lysis can be applied to selectively remove eukaryotic cells from the sample. This approach was used to separate bacterial ATP from biological fluids by lysing somatic cells with detergent as Triton X 100 at low concentration and combining this step with an enzymatic degradation of ATP released from lysed cells (Chapelle et al., 1978). RMDS protocol is based on sample filtration through membrane which naturally helps to eliminate extracellular ATP. If background ATP remains after filtration, rinsing the membrane with physiological serum or sterile water contributes to removal of residual ATP and allows bacterial detection. The figure 5 shows the impact of adding rinsing steps to reduce background on beverage products.

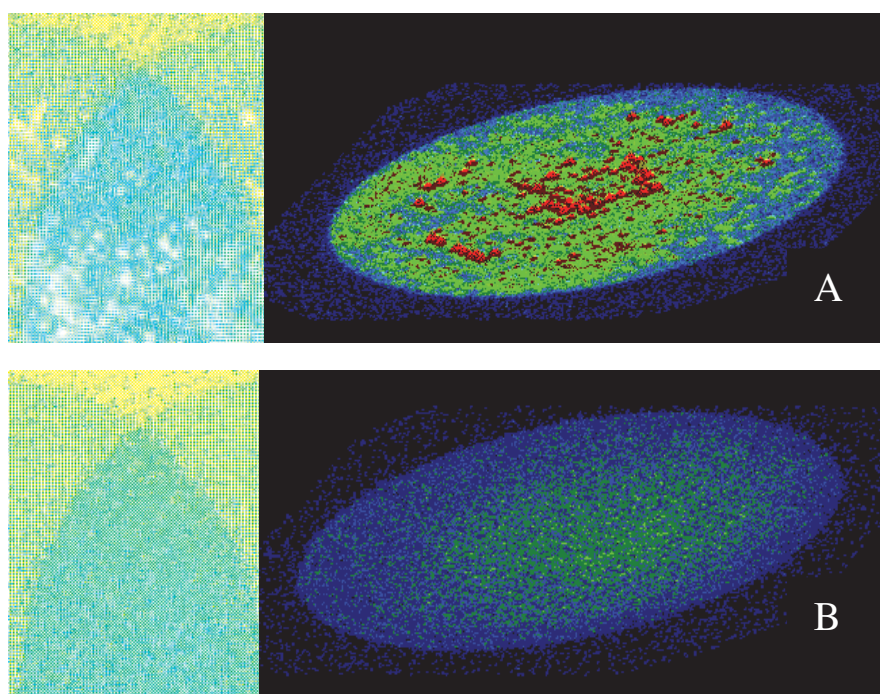


Fig. 5. Example of 2D and 3D views given by RMDS software for flavored water analysis with and without rinsing with sterile water. Picture A shows light spots corresponding to ATP present naturally in the filtered sample. Picture B shows the impact of rinsing water to remove background.

A protocol was developed to use RMDS to detect and quantify bacterial contamination from a mixture of mammalian cells and bacteria. The filtration of mammalian cells and bioluminescence detection through RMDS protocol shows (see Fig.6A) a high amount of light produced by mammalian cells preventing any bacterial detection. The sample treatment with a combination of a mammalian cells lysis solution and with apyrase contributes to efficiently remove the bioluminescent background and the figure 6B demonstrates that light spots remain detectable. These spots correspond to light emitted by bacteria in the mixture. Results obtained show that ATP-bioluminescent assay could be a powerful tool to microbiologically and quickly monitor eukaryotic cell cultures.

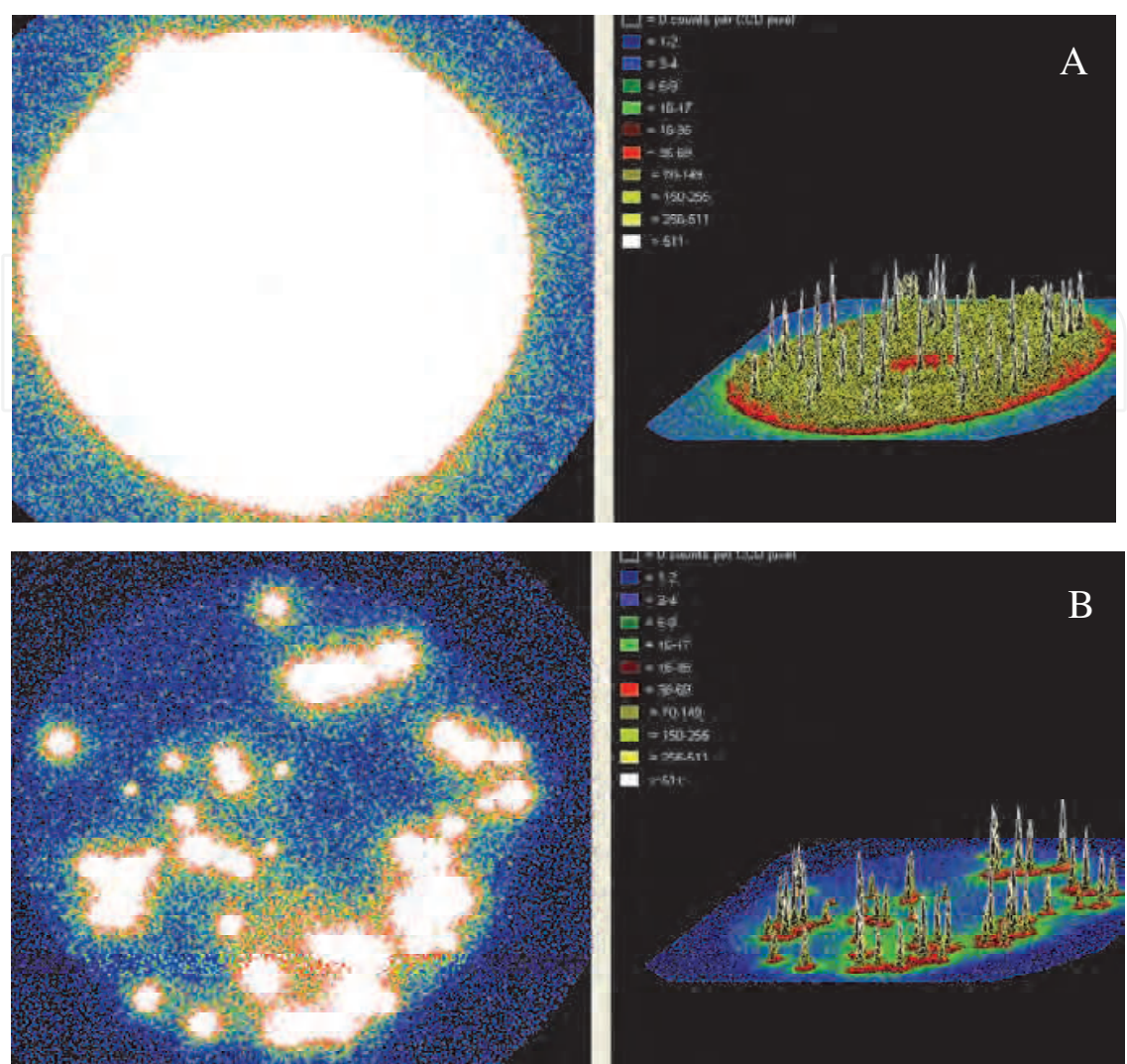


Fig. 6. A) RMDS analysis of 1mL of Chinese Hamster Ovary cells at  $10^6$ cell/mL. Eukaryotic ATP content generates a high bioluminescent background. B) RMDS analysis of a sample containing Chinese Hamster Ovary cells at  $10^6$ cell/mL contaminated with *E. coli* pretreated with a mammalian cells lysis solution and with apyrase. The sample pretreatment induces ATP background removal allowing contaminant detection.

### 3.5 RMDS applications

#### 3.5.1 Use of Bioluminescence for microorganisms detection in water

Water is a key raw material utilized in the manufacturing of products within the food and beverage, healthcare, microelectronics and pharmaceutical industries. Within each industry, different regulatory requirements exist for microbial contamination in the water used for the manufacturing of a product for a specific application. The microorganisms found in these water systems are mainly stressed, slow-growing strains characterized by long incubation times before growth can be detected using traditional microbiology methods such as membrane filtration or pour plates. The time it takes before contamination can be detected in water can cause delays in product release, and extend the storage time of products. Using a rapid Bioluminescence based detection method allows manufacturers to identify microbial

contamination earlier, which provides them with better process control, product yield, and shortens time to market.

The following table 3 provides the incubation times for detectable growth, by organism, for the traditional microbiology method and RMDS. The detection time is significantly reduced using RMDS. Detection of growth is on average 4.5 times faster than traditional microbiology, and up to 6 times faster for the very slow growers tested (*Methylobacterium mesophilicum* ATCC 29983, stressed strain of *Methylobacterium* and a mix of various slow-growing strains). RMDS allows for overnight detection of the industrial-stressed microorganisms tested. The incubation temperature also has an influence on time-to-result. Incubating at 25 °C showed that longer incubation times were required (data not shown). The mean recovery between RMDS and the traditional microbiology method in these experiments was 92.7%, which shows the equivalence of the two methods.

Microorganisms	Traditionnal Microbiology 30°C			Milliflex Rapid Detection System 30°C		
	R2A	PCA	TSA	R2A	PCA	TSA
ATCC Strains						
<i>P. aeruginosa</i> ATCC 9207	1 day	1 day	1 day	9 hrs	9 hrs	9 hrs
<i>M. mesophilicum</i> ATCC 29983	6 days	6 days	MNA	26 hrs	26 hrs	MNA
<i>E. coli</i> ATCC 8739	1 day	1 day	1 day	6 hrs	6 hrs	6 hrs
<i>B. cepacia</i> ATCC 25416	ND	ND	2 days	ND	ND	16 hrs
<i>S. epidermidis</i> ATCC 12228	ND	ND	1 day	ND	ND	9 hrs
Industrial-Stressed Microorganisms						
Mix of various slow-growing strains	6 days	6 days	MNA	24 hrs	24 hrs	MNA
Stressed strain of <i>Methylobacterium</i>	6 days	6 days	MNA	24 hrs	24 hrs	MNA
Environmental isolate of <i>R. pickettii</i>	2 days	2 days	ND	11 hrs	11 hrs	ND
MNA : Medium Not Appropriate for growth of microorganism, ND : Not Done						

Table 3. Detection time of reference strains and water isolates in traditional method and RMDS using either R2A agar, Tryptic Soy Agar (TSA) or Plate Count Agar (PCA).

3.5.2 Rapid detection of spores

Spores are major food spoilages and are also a concern in pharmaceutical samples. The classical microbiological method to enumerate spore contamination combines heat shock and on average 5 days incubation into sterile and molten specific medium Agar (Wayne et al., 1990). The amount of ATP in spores is very low and germination is necessary to increase ATP content and develop a rapid detection method based on ATP-bioluminescence (Kodata et al., 1996). ATP-bioluminescence rapid screening assay has been described showing that after germination, spore containing powder has been detected in a short time with a detection limit of 100 spores (Lee & Deininger, 2004). Fujinami et al (2004) also showed that short incubation of the sample in nutrient broth medium containing L-alanine increased RLU from spores and optimize the ATP- bioluminescent assay.



An easy protocol was developed to quickly enumerate spore contamination in artificially inoculated products with RMDS. Physiological water was inoculated with a calibrated concentration of *Bacillus subtilis* spores. After a heat shock at 80°C for 10 min, the inoculated product followed the protocol described in section 3.3. The incubation was performed with R2A medium at 32.5°C $\pm$  2.5°C. Results show that ATP bioluminescent signal start to be detected after 4h of incubation and that the reliable detection and enumeration of spores was achieved after 5 hours. Results given by RMDS are consistent with the expected inoculation level of the product and exhibit a recovery of almost 100% (tests performed in triplicate) compared with the control plate incubated 48h. Figure 7 gives an example of spore detection after 5h of incubation with R2A medium. RMDS protocol provides an alternative approach to perform rapid detection of spores in filterable matrix.

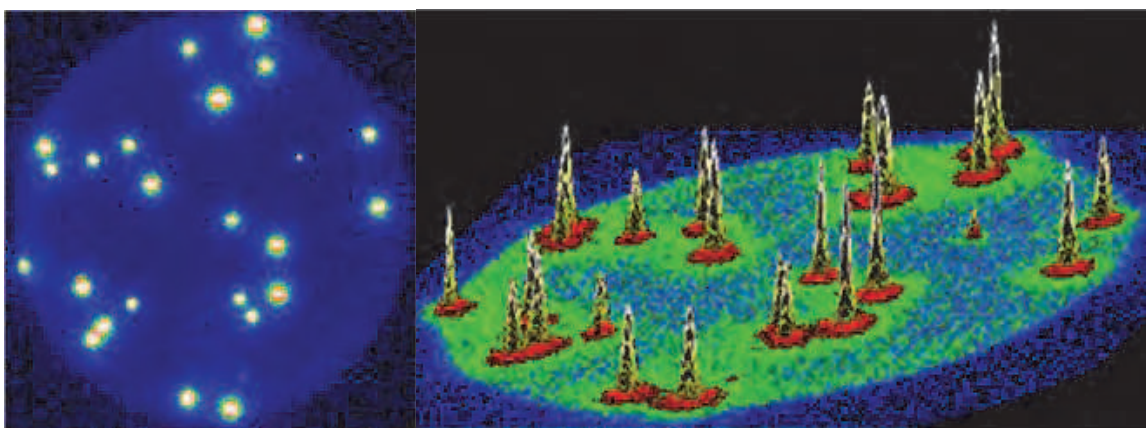


Fig. 7. RMDS 2D and 3D views showing *Bacillus* spore detection and enumeration.

### 3.5.3 Use of RMDS for the rapid detection of contaminants in bioreactor samples

RMDS has also been evaluated to detect contaminants in complex matrix containing mammalian cells. Mammalian cells including hybridoma are widely used in the biotechnology industry. Cell culture batches as well as consecutive downstream processes must be thoroughly monitored for microbial contamination. The ATP-bioluminescence technology is not selective of microbial ATP. The mammalian ATP released from the cells produces an interfering signal that must be eliminated to allow accurate counting of cfus.

Triton X100 combined with ATPase was already described to selectively extract and degrade ATP from blood products and urine samples enabling specific bacterial detection (Thore et al., 1985).

A simple and fast pretreatment method based on a selective lysis of mammalian cells and ATP removal has been developed. The harmlessness of this treatment for microorganisms was demonstrated, allowing the use of the RMDS to monitor mammalian cell samples. The protocol is as follow: 1. Differential lysis of the mammalian cells (Chinese hamster ovary [CHO]-K1, ATCC CCL-61) using the selective mammalian cell lysis solution (Millipore MSP010053); 2. removal of mammalian ATP using 5U apyrase (Sigma Aldrich); 3. Milliflex Rapid membrane filtration using Milliflex funnel; 4. phosphate buffered saline rinsing to remove remaining mammalian ATP; 5. membrane incubation for bacteria growth; and 6. detection and counting of bacteria using the RMDS as described in the protocol.



This method enabled the detection of microorganisms in the presence of up to  $5.10^7$  eukaryotic cells, and involved a single pre-treatment step of the sample prior to filtration. Figure 3 (paragraphe 3.2) demonstrates that in *E. coli*-contaminated CHO cells, the pre-treatment removed specifically mammalian ATP and enabled the enumeration of contaminants.

The harmlessness of the cells treatment toward microorganisms was also demonstrated using *B. subtilis*, *S. aureus*, *P. aeruginosa* and *Candida albicans* spiked at approximately 50 cfus with a recovery ranging from 80% to 109.7% compared with traditional microbiology counts. During the filtration step, mycoplasma, unlike bacteria, will pass through a 0.45  $\mu\text{m}$  filter (Baseman & Tully, 1997). Moreover, mycoplasma membranes are easily solubilized by detergents, and the lysis of mammalian cells simultaneously affects mycoplasma viability.

The specific mammalian cell lysis solution coupled with the RMDS method allowed fast detection of contaminating microorganisms in high value cell samples. Using RMDS to detect and quickly enumerate microbial contamination in biotechnology samples such as eukaryotic cells will allow better control throughout the process.

### 3.5.4 Rapid sterility testing based on ATP-Bioluminescence

In pharmaceutical companies, products are released based on microbiological quality. The Sterility test is a mandatory and critical step to ensure that the product is free of microorganism. The test takes 14 days of incubation before getting results. Time is the main reason why there is a need for an alternative and rapid method. Due to its universality and high sensitivity, the ATP-bioluminescence technology represents an alternative to ease sterility testing and shorten incubation time (Bussey & Tsuji, 1986). In addition to reduce time to detection, ATP-bioluminescence brings a solution to one drawback of current methods. Light detection replaces the subjectivity of visual determination of turbidity. Bioluminescence test that uses adenylate kinase reaction to convert ADP in ATP to significantly amplify the signal is described as a rapid sterility alternative method with results below or equal to 7 days (Albright, 2008).

Sterility testing based on RMDS follows the protocol described in section 3.2 with the major difference that the filtration step is performed under an isolator or a sterile chamber to ensure a sterile environment throughout the test. Reducing the incubation time from 14 days to 5 days is an achievable goal which benefits pharmaceutical companies. As RMDS is based on filtration, this method is compatible with complexe matrices. A comparative study was performed between RMDS and technologies based on CO<sub>2</sub> detection. Peptone water and biological matrix such as inactivated influenza vaccines were inoculated with low concentration of microorganisms representing Gram negative, Gram positive, aerobic, anaerobic, spore forming, slow growing bacteria, yeast, and fungi. Results showed that RMDS detected all microorganisms significantly faster than the compendial method (Parveen et al., 2011). RMDS using incubation onto Schaedler Blood Agar detected all tested microorganisms in 5 days in the presence of a matrix containing preservative 0.01% thimerosal and was also compatible with inactivated influenza vaccines and aluminum phosphate or aluminum hydroxide adjuvants (Parveen et al., 2011). RMDS is likewise used as rapid sterility testing by other pharmaceutical company and shows no interference with bioluminescence mechanism and a detection in 5 days of stressed and reference strains including worst microorganism such as *Propionibacterium acnes* (Gray et al., 2010).

### 3.5.5 Use of RMDS for specific detection of *Pseudomonas aeruginosa*

#### 3.5.5.1 Specific detection protocol

RMDS was used with specific hybridization probes targeting *P. aeruginosa* and coupled to Soy Bean Peroxydase. A new and unique permeabilization solution was developed and is based on polyethylimine (PEI). Cells are fixed on the membrane using a formaldehyde mix. Hybridization was performed using Peptide Nucleic Acid probes targeting 16S RNA conjugated to Soybean peroxydase diluted in Hybridization buffer. Free probes are washed with a Tween buffer. SBP catalyzes conversion of Luminol into photons and light activity of the bioluminescent reaction is recorded by the CCD camera of RMDS.

The following procedure was used to determine the minimum incubation time necessary to detect and enumerate *P. aeruginosa* with RMDS (Fig 8):

1. Pour 50 mL of saline solution into a Milliflex funnel; 2. Spike the appropriate dilution of each microorganism into the funnel (10–100 CFUs); 3. Add 50 mL of saline solution into the funnel to homogenize the content; 4. Filter and transfer the membrane onto a prefilled TSA Milliflex cassette. Incubate at  $32.5\text{ }^{\circ}\text{C} \pm 2.5\text{ }^{\circ}\text{C}$  for the appropriate time; 5. Once incubation is complete, separate the membrane from the cassette and let the membrane dry; 6. Follow the Milliflex Rapid *P. aeruginosa* detection procedure described before; 7. Spray the specific detection reagents using the Milliflex Rapid AutoSpray Station; 8. Read the sample with the Milliflex Rapid Detection and Enumeration System. Steps 1 through 4, 6 and 8 were performed inside a laminar flow hood.

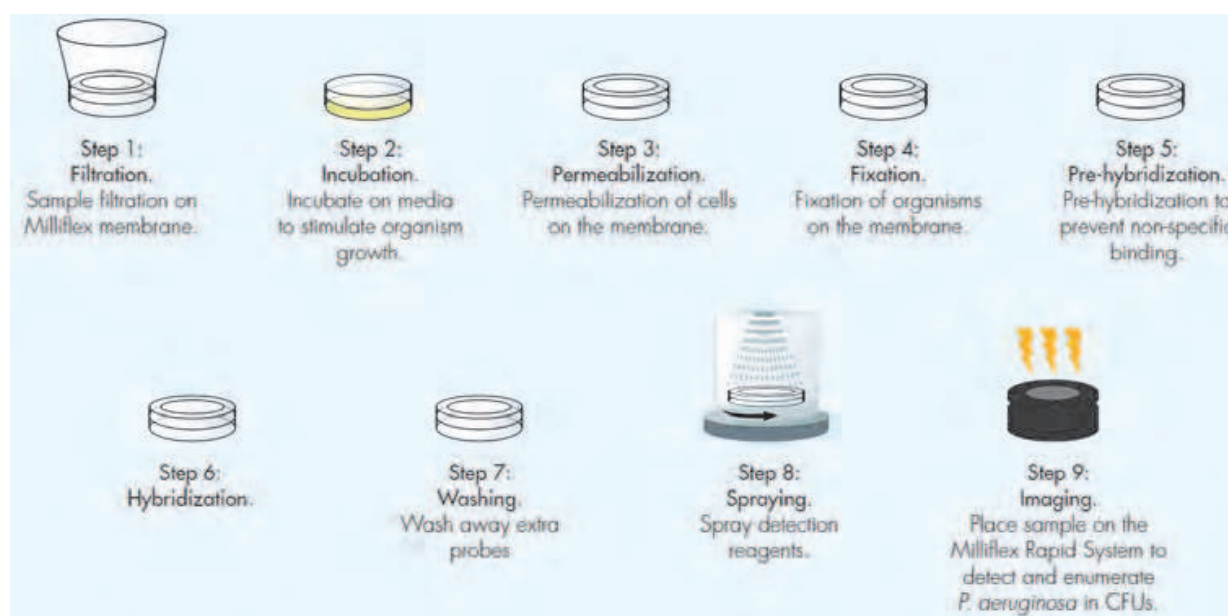


Fig. 8. *P. aeruginosa* specific detection and enumeration protocol

This procedure was also used to obtain both total viable count (TVC) and specific detection and enumeration of *P. aeruginosa* using the same membrane sample. The adapted procedure is as follow:

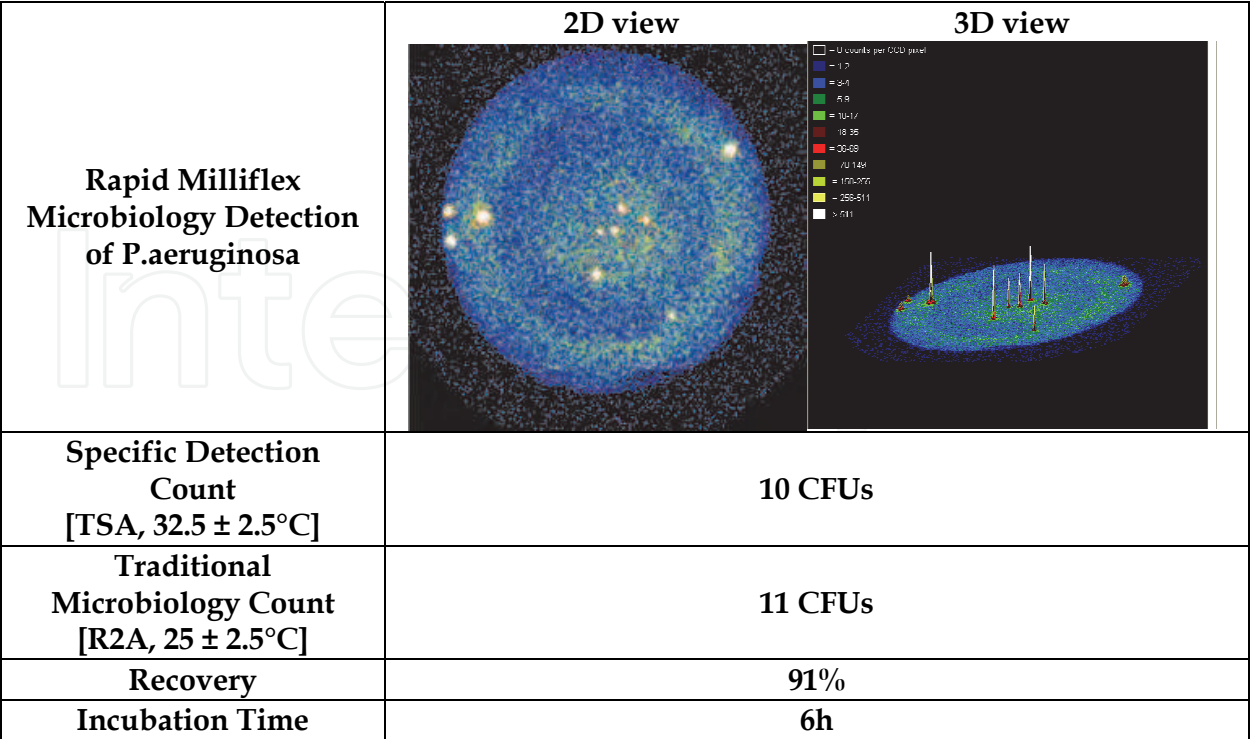
pour 50 mL of saline solution into a Milliflex funnel. Spike the appropriate dilution of each microorganism into the funnel (10–100 CFUs). Add 50 mL of saline solution into the funnel

to homogenize the content. Filter and transfer the membrane onto a pre-filled TSA Milliflex cassette. Incubate at  $32.5\text{ }^{\circ}\text{C} \pm 2.5\text{ }^{\circ}\text{C}$  for the appropriate time. Once incubation is complete, separate the membrane from the cassette and let the membrane dry. Spray the ATP releasing and bioluminescence reagents using the Milliflex Rapid AutoSpray Station. Read the sample with the RMDS. Then, follow the Milliflex Rapid *P. aeruginosa* detection procedure starting from fixation step. Spray the specific detection reagents using the Milliflex Rapid AutoSpray Station and read the sample with the Milliflex Rapid Detection and Enumeration System.

3.5.5.2 Specific *Pseudomonas aeruginosa* detection and total viable count results

The Milliflex Rapid system is a proven automated solution for the rapid detection and enumeration of total viable count (TVC) in purified water and Water For Injection. Based on membrane filtration and image analysis together with an adenosine triphosphate (ATP) bioluminescence reagent, the Milliflex Rapid System delivers TVC test results faster than traditional methods. We have developed a hybridization assay that enables the Milliflex Rapid system to specifically detect and enumerate *P. aeruginosa*. The hybridization assay is performed with a peroxidase-conjugated DNA-oligonucleotide probe targeted to a specific RNA-sequence of *P. aeruginosa*. Applying luminol and peroxide substrates to the membrane filtration sample generates light that is detected by the Milliflex Rapid system.

In order to determine the minimal incubation time to detect *P. aeruginosa*, a pure culture of *P. aeruginosa* ATCC 9027 was spiked into Milliflex and incubated on TSA for 6 hours for the alternative method and on R2A for 24 hours for the compendial method. Results are presented in Figure 9.



Using the specific detection procedure described above *P. aeruginosa* was detected and enumerated in 8 hours in a water sample. The specificity of the method has been assessed against numerous microorganisms and only *P. aeruginosa* was detected in this panel of contaminants. The limit of the sensitivity is 1 CFU (data not shown).

The objective of this experiment was to first obtain the TVC in CFUs using the total viable count assay, followed by the specific detection assay for *P. aeruginosa*. After performing the TVC analysis, the results were stored on the Milliflex Rapid system and the same membrane was then treated following the specific detection procedure. The TVC and the specific detection count data were then analyzed (fig.10).

Figure 10 provides results for both TVC and specific detection of *P. aeruginosa* using the same membrane. The images below show that the position of each colony forming unit is identical when using the TVC and specific detection assay. One hundred percent of the CFUs were detected in each assay.

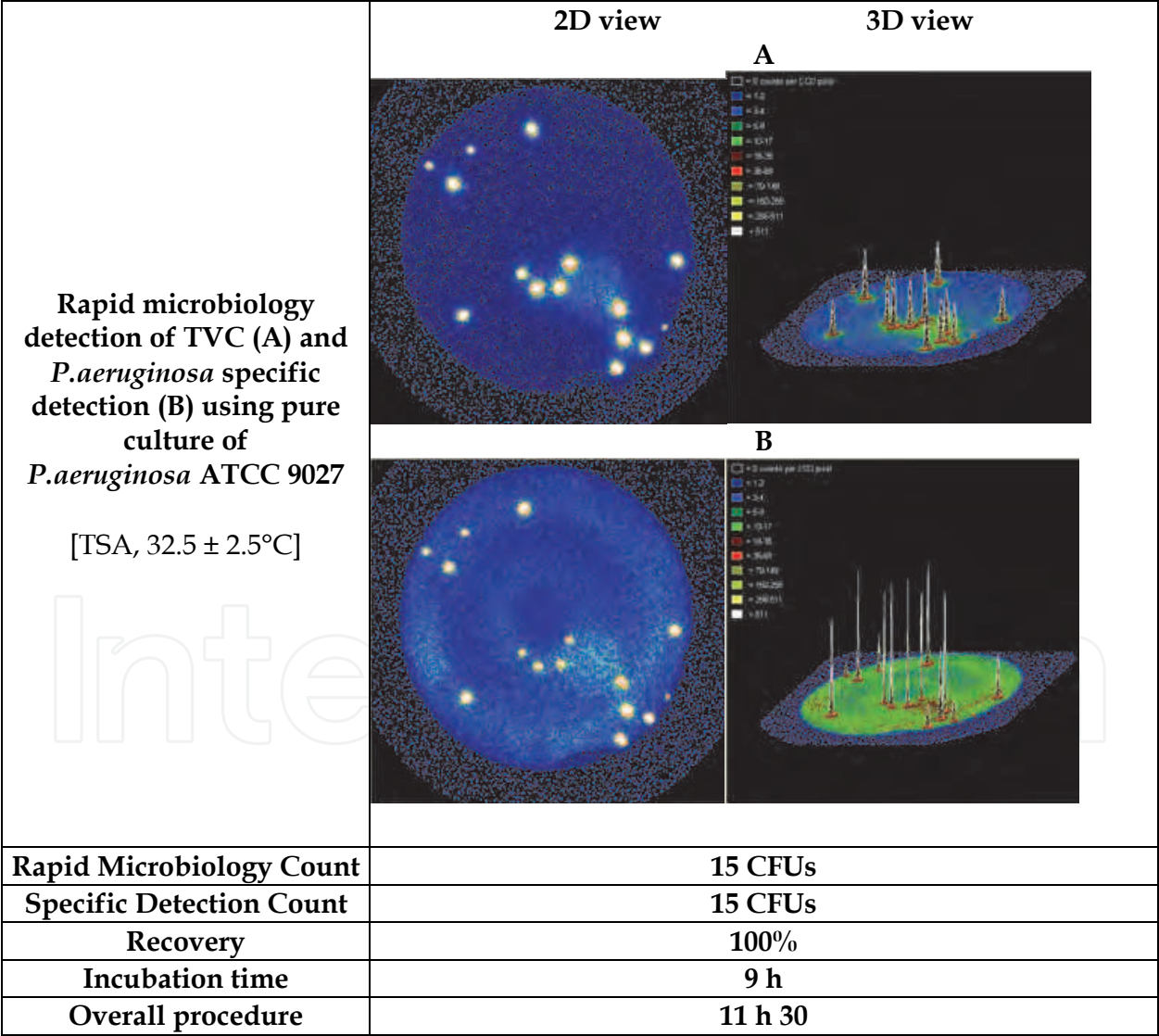


Fig. 10. Specific detection of *P. aeruginosa* after TVC on the same membrane using pure culture of *P. aeruginosa* ATCC 9027 incubated on TSA at 32.5°C+/-2.5°C.



In a second assay, a mixed microbial population composed of *P. aeruginosa*, *Burkholderia cepacia* and *E. coli* were spiked and analyzed with the procedure described in “Combination of Total Viable Count and Specific Detection of *P. aeruginosa*.” Results are presented in the figure 11. After 9 hours growth at 35 °C, 24 CFUs were detected after the TVC procedure and 8 CFUs were detected using the *P. aeruginosa* specific detection procedure.

This demonstrates that the system is able to make TVC and specific detection even in a mixed population of microorganisms.

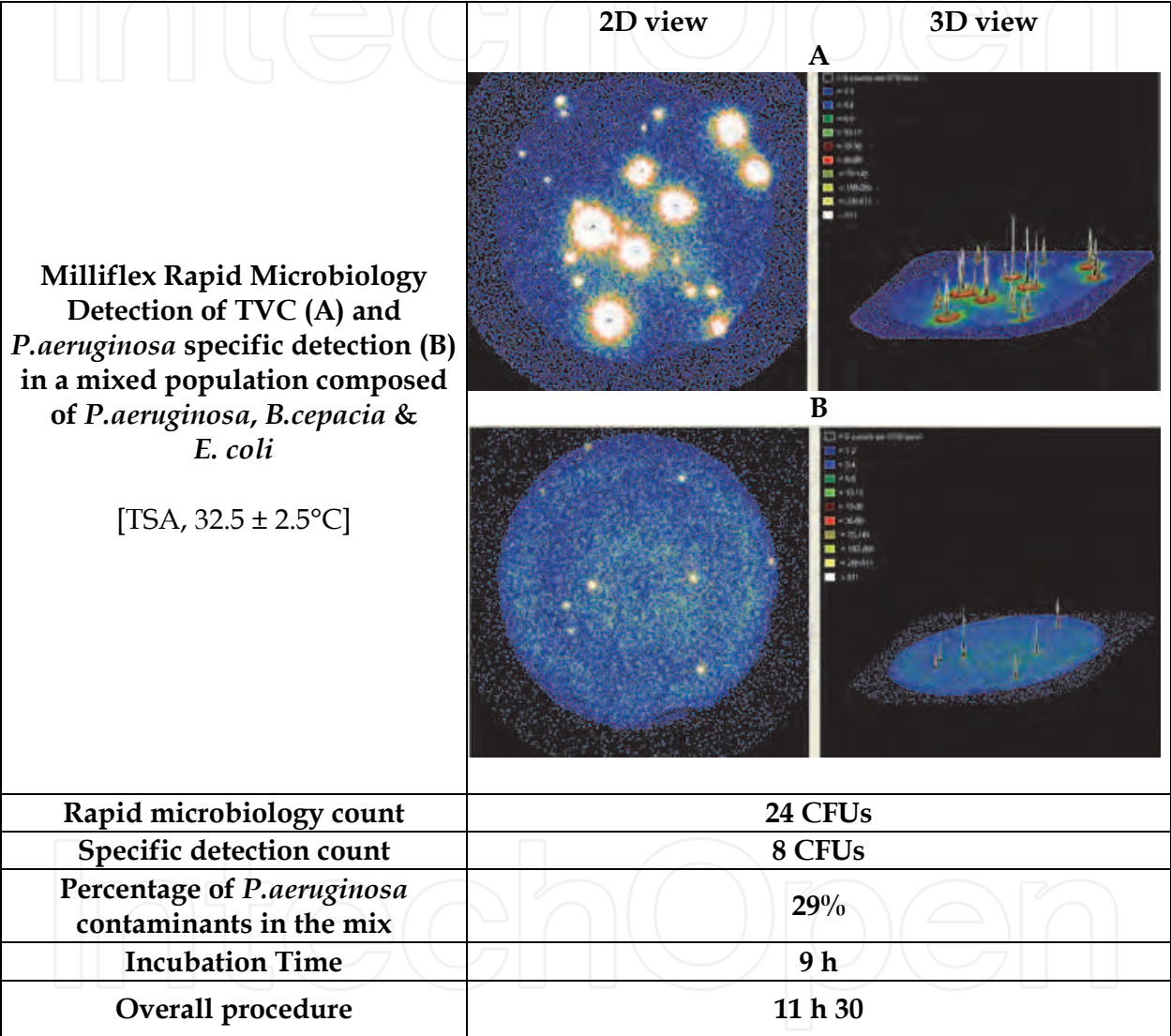


Fig. 11. Specific detection of *P. aeruginosa* after TVC on the same membrane using a mixed population of *P. aeruginosa* ATCC 9027, *B. cepacia* ATCC 25416 and *E.coli* ATCC 25922 incubated on TSA at 32.5°C+/-2.5°C.

4. Conclusion

The different studies presented here show how versatile is the use of Bioluminescence for microorganisms detection. We demonstrate here that it offers a high sensitivity to detect microbial contamination rapidly in a variety of filterable samples.

The association of Bioluminescence to sensitive sensors such as RMDS provides a result in colony forming units equivalent to the standard plate count but is 4 times faster than classical microbiology. This method can be used in samples from industrial water, to food and beverage samples for the detection of any type of bacteria, yeasts and molds including spores. We also showed that it can be used to detect bacterial contamination in cell culture matrices containing high concentrations of eukaryotic cells.

Interestingly, Bioluminescence was also coupled to molecular biology through the use of 16S RNA probes for specific detection of bacteria. The example presented here allowed not only the detection of *P. aeruginosa* but also the total viable count using Luciferin and luciferase followed by specific detection of this very specific bacterium.

Finally, the development of the method in a pharmaceutical environment allowed sterility testing of drug products 3 times faster than the compendial method. This recent developments in the pharmaceutical field show that the method is also able to help patients taking drugs usually associated with a very short shelf life (gene therapy products, cell therapies...) as the result is delivered before the injection of the product while the traditional systems usually deliver after the treatment.

In conclusion, the use of Bioluminescence either in its "classical" or molecular format allows for a number of developments in the field of microorganisms detection. The flexibility of the method and its ease of use coupled to the considerable savings in time compared to the traditional method make it a valuable tool for life scientists as well as for other clinical applications.

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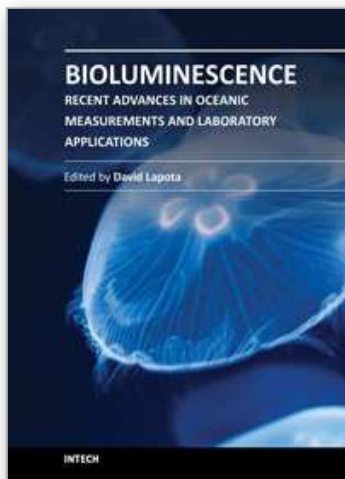
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## **Bioluminescence - Recent Advances in Oceanic Measurements and Laboratory Applications**

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We now find ourselves utilizing luciferase - luciferin proteins, ATP, genes and the whole complex of these interactions to observe and follow the progress or inhibition of tumors in animal models by measuring bioluminescence intensity, spatially and temporally using highly sophisticated camera systems. This book describes applications in preclinical oncology research by bioluminescence imaging (BLI) with a variety of applications. Chapters describe current methodologies for rapid detection of contaminants using the Milliflex system, and the use of bioluminescence resonance energy transfer (BRET) technology for monitoring physical interactions between proteins in living cells. Others are using bioluminescent proteins for high sensitive optical reporters imaging in living animals, developing pH-tolerant luciferase for brighter in vivo imaging, and oscillation characteristics in bacterial bioluminescence. The book also contains descriptions of the long-term seasonal characteristics of oceanic bioluminescence and the responsible planktonic species producing bioluminescence. Such studies are few and rare.

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