Numeration methods for targeting photoactive materials in the UV-A photocatalytic removal of microorganisms

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This *tutorial review* reports on the different numeration methods for evaluating the efficiency of the photocatalytic action on microorganisms. Here we put forward the advantages and drawbacks of the standard methods such as the plate count, the fluorescence techniques and the Most Probable Number method for determining the biocidal photocatalytic activity and thus selecting efficient photocatalytic materials among complex systems. We highlight that bacterial spores are a representative and suitable tool for meeting the restrictive requirements resulting from the complex use of living matter instead of chemical targets.

1. Introduction

Photocatalysis by polycrystalline semiconductor oxides is a relatively recent catalytic technology, in which activation of the catalytic solid occurs through photon absorption, for irradiation wavelengths greater than the semiconductor band-gap (mainly in the near UV, *i.e.* UV-A). Transfer of photo-generated electrons and holes from the light-activated bulk to the photocatalyst surface allows redox reactions to occur with adsorbed reactants (Scheme 1). Photooxidation is the most popular class of photocatalytic reactions, leading to mineralization of organics, *via* the oxidative photogenerated holes or the creation of highly reactive surface radicals such as OH°.[1]

The first report of Matsunaga *et al.* in 1985 on the microbiocidal effects of TiO_2 opened the door to a growing crossing-over between photocatalysis and life science.[2] They studied the efficiency of photocatalytic oxidation of *Saccharomyces cerevisiae* (yeast), *Lactobacillus acidophilus* and *Escherichia coli* (bacteria), and *Chlorella vulgaris* (green algae) in water, and showed the killing of microbial cells using a Pt–TiO_2 photocatalyst. They proposed that the root cause is the photooxidation of terminal sulfhydryl groups of intracellular Coenzyme A, participating in many enzymatic reactions involved in the respiratory chain, leading to cell respiration inhibition and finally to death. This gave rise to extensive research in the field of oxidative photocatalysis, mainly dealing with TiO_2 suspensions, and targeting bacteria, viruses, fungi, algae and protozoa.

The mechanisms for cell killing remain under debate, and are not reviewed here. Regardless of the oxidative species involved, there is substantial evidence that contact with TiO_2

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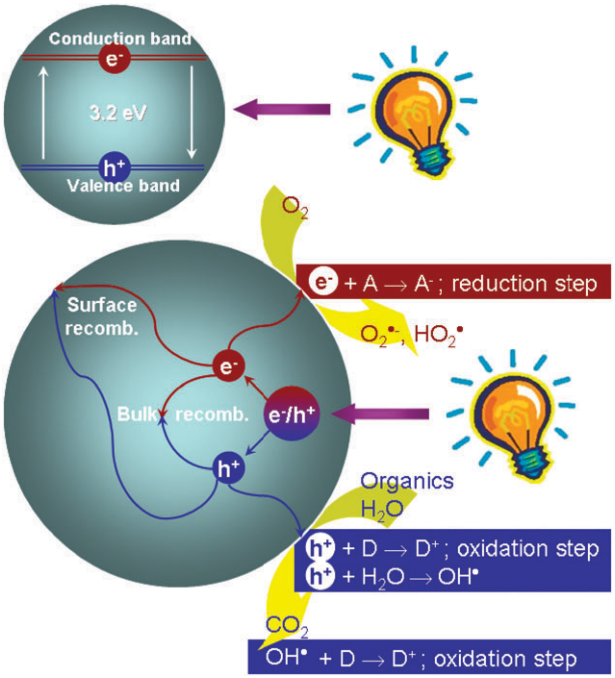

Scheme 1 (Top) Activation of a semiconductor particle with promotion of an electron from the valence to the conduction band, with the simultaneous creation of a photogenerated hole within the valence band. (Bottom) Schematic evolution of a photogenerated electron/hole pair within a light-activated semiconductor particle. At the particle surface: the redox reactions are separated into reduction and oxidative steps, involving on one hand, conduction band electrons and adsorbed electron acceptors following $e_{CB}^- + A \rightarrow A^-$ (O_2 playing the role of electron acceptor), and on the other one, valence band holes and adsorbed electron donors following $h_{VB}^+ + D \rightarrow D^+$ (organics playing the role of electron donor). Water can also be oxidized by photogenerated holes to create highly oxidative OH° hydroxyl radicals, further directly participating in the mineralization of organic molecules into CO_2 and H_2O. For more details: see ref. 1.

causes oxidative damage to the cell membrane. Well-documented reviews provide a clear overview of the application of TiO_2 photocatalysis to disinfection or killing of cancer cells.[3]

In contrast to photocatalysis applied to chemicals, which has for decades attracted much interest in liquid and gas phase processes, the application of photocatalysis to the removal of microorganisms remained mainly focused on the treatment of liquids and surfaces. For liquid applications, this resulted from extensive contamination of surface and groundwater by industrial effluents, excessive use of pesticides, and domestic waste landfills. Up to now, the processes most used for disinfecting fluids are chlorination, ozonation and germicidal UVC lamps (low pressure Hg vapor lamps emitting at 254 nm) for water treatment while size exclusion filters, germicidal lamps, thermal treatments and disinfection using chemical agents are used for air decontamination. Filtration usually implies high costs, due to the micrometric size of the biological species. This recuperative process requires a post-degradation treatment to kill the bacteria. Moreover, the short lifespan of filtration systems is restrictive and thermal-chemical treatments require compulsory isolation of the contaminated zone during disinfection. Photocatalysis applied to biological targets is thus a promising alternative. The public concern over human health and water potability has created a strong incentive and is driving research in this area, explaining the growing interest in the development of new light-driven processes for water disinfection. Recently, Reed reviewed solar disinfection as a water treatment process, and Rincon and Pulgarin detailed the Fe^{3+} and TiO_2 solar light assisted inactivation of *E. coli* at field scale for treating large quantities of water.[4]

By contrast, work on the photocatalytic disinfection of contaminated air by pure UV-A photocatalysis remain scarce, despite the great interest for public health reasons and a large spectrum of applications. Little work has been published on removing bacteria from humid air, and the first report seems to be that of Goswami *et al.*[5] Recent communications were devoted to the treatment of flowing air contaminated by *E. coli* and *Legionella pneumophila* bacteria, T2 bacteriophage viruses and *Bacillus subtilis* spores.[6]

Here, we show the specificities and the restrictions relative to the investigation of microorganisms instead of the usual chemicals as targeted agents. Many parameters acting significantly at the biological level and not taken into account for chemicals are reviewed, in order to highlight the interest of using spores as a valuable tool. Numeration methods for evaluating the photocatalytic behavior of materials towards living matter and targeting suitable complex photocatalytic systems, are reported, including the plate count (spread plate, pour plate and spiral plating), the fluorescence techniques (cytometry and epifluorescence microscopy) and the Most Probable Number (MPN) method. Crucial metrological problems are described, putting forward the drawbacks, advantages and specificities to be taken into account for each method, since inappropriate counting techniques (or an inappropriate way of processing an adequate technique) undoubtedly present the microorganism inactivation results in a wrong way, especially in an inter-disciplinary field with different approaches.

2. Living matter *vs.* chemical targets in photocatalysis

The analogy between chemical and biological targets results from the organic nature of the microorganism constituents, that photocatalysis can oxidize—mineralize—similarly to liquid and gas phase organics during *e.g.* potabilization or depollution oxidative processes for water or air treatment. For instance, depending on the cell nature, the cell walls—the first surface target of photocatalysis—are made of a complex assembly of high molecular weight compounds (M_W > 10 000), such as peptidoglycan polymers, teichoic acids, lipopolysaccharides, lipoproteins, phospholipids, even cellulose for fungi, or sterols for spores (Fig. 1).

This analogy results in similar research trends, linked to the search for new materials in terms of activation and photogenerated charge separation. Although TiO_2 is currently the most attractive and efficient photocatalyst for UV-A activation, with a 3.2 eV band gap energy requiring a near UV light, the search for new photocatalytic materials is of high interest for:

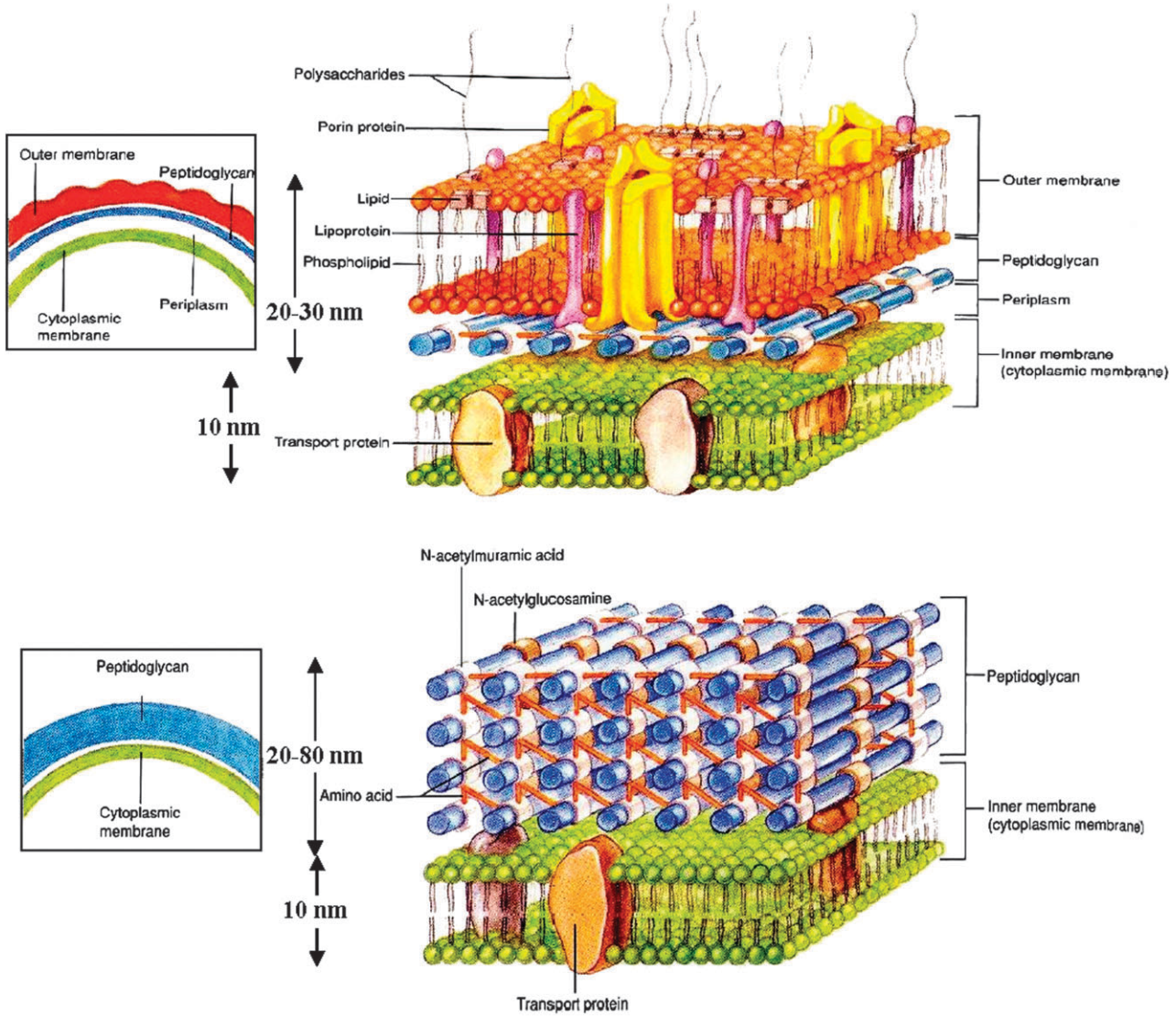

Fig. 1 Schematic views of Gram negative (top) and Gram positive (bottom) bacteria cell walls, showing the complex assembly of high molecular weight compounds.

— improving the photooxidation activity under UV-A illumination. The efficiency of photocatalytic materials is limited by the recombination of photogenerated charges and by adsorption–desorption problems of both reactants and reaction products. These key-points are investigated by modifying pure TiO_2 anatase and/or designing more complex photocatalytic systems.

— transferring the activation mode from the UV-A to the visible-light range. Since UV occupies only about 4% of the solar spectrum, transferring the activation mode to visible light, either using artificial lighting, or as a first step towards the solar *Grail*, is of high importance for sustainable development and remains a major challenge in which much of research is being conducted. The approaches for achieving this goal are focusing on the material design at the nano-level.

The discovery of new photocatalytic materials with enhanced performances is therefore one of the driving forces for modern photocatalysis whatever the target nature, and research has to take into account the design of new photocatalytic solids. Many routes are studied, like *e.g.* doped TiO_2, TiO_2 with ad-atoms, defined structured semi-conductors, sensitized or grafted materials, new morphologies of photocatalysts, titanium-free materials and coupled binary or even ternary complex systems.

Such modifications have been *quasi*-exclusively studied for chemical molecules and the range of material modifications remained very narrow for biological targets. The main studies concern the modifications of TiO_2 photo-catalysts by addition of silver ions,[7] especially for using a synergistic effect provided by the location of Ag^+ ions at the TiO_2 surface in photocatalysis and the widely known anti-microbial properties of Ag^+ ions. To a lesser extent, other metals such as copper or platinum have been used with TiO_2.[2,8] Borkow and Gabbay provided a remarkable paper on the use of copper ions as bactericidal, virucidal, antifungal, and antimite agents.[9] But even in the case of silver—the main modification of TiO_2 reported in the literature—known as a disinfectant for centuries and with a broad spectrum of anti-bacterial activity, the

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exact mechanisms of the biocidal action are still not fully understood, although some have been proposed, from the late 70s up to recent work.[10,11]

Few other elements than copper and silver (we can list Pd, N, Nd, Au and Pt)[12] have been tested for their synergistic effects with TiO_2 for disinfection, by contrast to the extensive literature available on the degradation of chemicals. The reason for this relative lack of studies concerning the improvement of biocidal photocatalysts results probably from an experimental work being much more difficult, complicated and subject to far more variability than that with chemical targets.

The analogy between chemicals and living matter in photocatalysis stops here, as such studies must take into account new specific parameters operating at the biological level. Such studies remain absolutely necessary for improving the efficiency of photocatalytic disinfection and implementing the mechanistic understanding, as detailed in the further sections.

3. Influent parameters at the biological level

Many parameters influencing the efficiency of the photocatalytic degradation of chemicals are well established and reviewed.[1] Some of them directly concern the catalyst itself, like the surface area, the pore size distribution or the crystal size, easily measurable and to some extent controllable. Parameters linked to the experimental conditions, such as the temperature, the reactant concentration or partial pressure, as well as the amount of adsorbed O_2 or H_2O can also drastically influence the photocatalytic efficiency over chemicals. However, it seems that the most difficult variable to be measured is the efficiency with which photons are absorbed and lead to a catalytic act.

In addition to these sources of variability, we point out that working with living organisms presents many other specific obstacles to be taken into account, that greatly increase the difficulty of the measurements and the requirements to be met. These influent parameters are detailed below.

3.1 The target microorganism

The nature of the microorganism is important, since as reviewed by Huang *et al.*,[13] sensitivity toward photocatalysis varies in the order: virus > bacterial cell > bacterial spore. Even for bacteria, and more specifically within the Gram group, Gram negative bacteria were more affected than Gram positive, as a result of structural differences in the outer membrane.[14]

The most studied microorganism in photocatalysis remains *E. coli*, undoubtedly because it is the reference microorganism in biology. However, the strain used is a crucial parameter. Indeed, within the strains of a species, almost all tested *E. coli* strains come from the genetically engineered non-pathogenic K12 which notably possesses about 15% less genetic material than one of the real targets causing diarrhoa, *E. coli* 0157:H7.[15] The amount of enzymes produced by the cells to protect them from oxidative stress may vary greatly and thus different efficiencies can be obtained.[16]

3.2 The growth conditions

It is quite obvious that varying oxidative conditions during the growth (mainly oxygen) might have a great influence upon the decontamination efficiency of a photocatalytic test since the presence of an oxidative component enhances the synthesis of the two main enzymes responsible for protection against the inevitable associated radicals. Bacterial agents grown over agar plates may respond differently from others grown in broth. In addition, for a given microorganism, the cultivation medium used could be a source of huge variability in sensitivity, as shown in another area.[17]

Another point is the growth temperature: since the cell wall composition seems to have a great influence in the decontamination process as described by Maness *et al.*,[18] variations of the chemical constitution of the outer membrane induced by the growth temperature could also act upon the removal rate of the contaminant.[19]

The impact of the culture replication was also evidenced, the sensitivity towards photocatalytic oxidation increasing with the replication number at which cells are harvested.[20] This phenomenon was mainly attributed to the mutations which may occur at each replication and which are subsequently transferred to the next. Phase changes can also be involved. Therefore, working with cultures from frozen aliquots and harvested at the first replication is recommended.

The physiological state of the cell is another factor, as confirmed by Verran *et al.*,[21] bacteria harvested during the exponential growth phase are more sensitive than cells which have reached the stationary phase. Cells in the exponential phase turn the whole metabolism torwards multiplication, thus leaving less capacity to the repair system. In contrast, once bacteria are in a stationary phase, they switch their metabolism to a "survival mode" and major differences appear in the outer membrane.

The presence of exopolysaccharides involved in the formation of biofilms may also negatively influence the sensitivity of the cells.

3.3 The experimental settings

Firstly, it is clear that the results depend greatly on the reaction media (air, liquid or surface treatments) as is also true for chemical applications. A microorganism displays a specific sensitivity toward its surrounding media: decontamination tests should take into account the natural environment of bacteria and thus should especially avoid the use of waterborne microorganisms for gas phase experiments.

Other sources of variation are due to the experimental settings, like the initial concentration of microorganisms, as reported by Rincon and Pulgarin for bacteria-contaminated liquid phase treatment.[20] High concentrations lower the efficiency because cell lysate absorbs UV light and also competes with viable cells for photocatalytic degradation.

However, the main concerns are for microorganism recovery at the end of a photocatalytic test to provide a viability count. On the one hand, bacteria are able to recover viability through two well-established mechanisms: dark repair and photoreactivation. It has been shown for two decades that *L. pneumophila* possesses one of the most efficient repair

systems.[22] Thus, since the viability count should usually be performed shortly after the experiment, the bacteria numeration will overestimate the process efficiency, by not taking into account the bacteria repair efficiency, which is highly dependent on the experimental conditions. On the other hand, osmotic shock combined with mechanical stress can artificially increase the efficiency,[23] and thus the viability counting. Starting with 10^4 CFU mL^{-1} *E. coli* poured into distilled water, the amount of viable *E. coli* decreased down to below the detection limit within one hour (CFU means Colony Forming Units, see section 5.1 for more details), while by contrast only 0.4 log reduction was obtained with physiological saline (containing 0.9 wt% NaCl). The delay between the end of an experiment and the viability count procedure has thus to be considered as an influent factor if distilled water is used. Physiological saline should thus be preferred to distilled water.

In addition, one should focus on the temperature and hygrometry parameters during the experiments. They directly act on the survival rate of bacteria and are, *e.g.* in bioaerosol treatment, the most important factors to be taken into account since they govern bacteria desiccation and aerosol stability.

4. The use of bacterial spores as a reference tool

We have established that working with living organisms is bound to variations that are not trivial to overcome and introduces specific parameters, and thus restrictive constraints, that do not need to be considered in chemical applications.

However, it is possible to mitigate and even to render these variations negligible by using bacterial endospores. These are a form of ''dormant bacteria'' mainly produced by Gram positive bacteria to overcome stress situations (*e.g.* starvation) and thus to wait for better conditions to germinate.[24] In this form, spores do not have a measurable metabolism and do not multiply. Their extreme resistance also makes them a useful reference tool for photocatalytic decontamination tests. As known for decades, spores can survive heat, γ-rays, H_2O_2, antibiotics and natural time decay: spores found in a 25 million year-old bee conserved in amber were able to germinate.[24]

This indicates that spore suspensions are stable for very long periods and have no susceptibility toward experimental settings such as humidity, temperature, mechanical stress or osmotic pressure. The spores which are usually used are those of the bioindicator *Bacillus subtilis* (recently reclassified as *Bacillus atrophaeus*).[25] No significant variation of the concentration in a *Bacillus atrophaeus* suspension was observed over sixteen months.[26] This lack of metabolism implies that no mutations can occur, and thus ensures a homogenous and constant genotype. In addition, there is no efficient repair system as in vegetative bacteria so that once damaged enough, spores cannot recover viability and germinate. Because of this, spore suspension can be considered almost as a chemical solution for performing tests.

Furthermore, endospores are a valuable tool in photocatalytic decontamination because the resulting efficiency will always be inferior to that obtained with vegetative bacteria as reported by Huang *et al.*[13] This explains why endospores are used in standardization testing of sterilization efficiency. Hot-air sterilization at 180 °C, considered up-to-now as the most efficient process, relies on the viable *Bacillus atrophaeus* spore count for norming a hot-air sterilization device to the DIN 58947 and DIN EN 866-6 standards. Working with spores of *Bacillus atrophaeus* dedicated to hot-air sterilization norms (such as the CIP 77.18, DSM 675 and ATCC 9372 strains), is therefore of great interest for obtaining representative, standardizable results, since titrated suspensions are commercially available. In addition, modelling of highly pathogenic anthrax spores can be perfectly performed using the non pathogenic *Bacillus atrophaeus* spores. The use of an easy to handle model is of great importance for performing tests and acquiring information and experience on such systems of high public concern.

5. The numeration methods

Using bacterial spores reduces the uncertainty of photocatalytic decontamination tests. Anyhow, imprecision in such biological applications remains a general metrological problem since the beginning of the 20th century. Considerable efforts have been made to enhance the accuracy of cell density measurement but there is still nothing comparable to the usual chemical precision. This section deals with some of the methods most employed, keeping in mind that a valuable count process expected to become a norm, should be on the one hand accurate, but on the other one easily and financially practicable. Here are detailed the plate count (spread plate, pour plate and spiral plating), the fluorescence techniques (cytometry and epifluorescence microscopy) and the Most Probable Number (MPN) method. The relative metrology as well as the drawbacks, advantages and specificities to keep in mind when using each method are described. A summarized comparison is reported in Table 1.

5.1 Plate count

Heterotrophic plate count (HPC)—formerly known as ''standard'' plate count—was introduced at the end of the 19th century by R. Koch for quantifying contamination in liquid samples. Still widely used, this method is based on the direct numeration of the colonies formed on a nutrient agar by cultivating an aliquot from the initial sample or one of its serial dilutions (Fig. 2). As it is not a direct observation of the metabolism of individual bacteria, the unit used remains the CFU (Colonies Forming Units) which is not directly a number of microorganisms. It should also be noted that the results obtained are not an accurate assessment of total organisms present.

They are only estimations of the number of organisms able to multiply on a given medium, for a given duration to form a colony. Wagner *et al.* estimated that only a small fraction of the total bacteria found using direct microscopy are enumerated using HPC procedures.[27] This can be explained by taking into account that the choice of medium is not always adapted or that two or more bacteria can form a single colony if they form clusters (like *Staphylococcus aureus* does) or if colonies merge to form a single one. In addition, not all viable bacteria will form a colony within the cultivation duration, especially

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Table 1 Summarized comparison between the main numeration methods

Methods		Advantages	Drawbacks
Heterotrophic plate count (HPC)	Spread plate	Widely used method (standardization interest)	Indirect count
		Little biological material necessary	Limited recovery–VBNC bacteria
			Deadline for reading results
			Merge of colonies
			Operator-dependent count
	Pour plate	Widely used method (standardization interest)	Indirect count
		Little biological material necessary	Limited recovery–VBNC bacteria
		Longer incubation time possible than in the spread plate method without colony merge (better recovery)	Deadline for reading results
			Not applicable to heat sensitive bacteria
			Operator-dependent count
			Expensive apparatus necessary
	Spiral plating	Non operator-dependent count	Indirect count
		Rapidity of the preparation	Limited recovery–VBNC bacteria
			Deadline for reading results
			Not applicable to heat-sensitive bacteria
			Operator-dependent count
Fluorescence	Epifluorescence microscopy	Direct count and	Many fluorochromes available (difficult interpretation)
		Rapid results obtained	
		Semi-automation possible (reading of the results)	Operator-dependent count (if manual count)
			Expensive apparatus necessary
			Decay of the fluorescence with time
	Flow cytometry	Direct count and rapid results obtained	Many fluorochromes available (difficult interpretation)
		Fully automated	
		Semi-automation possible (reading of the results)	Very expensive apparatus necessary
Most probable number	MPN	Widely used method (standardization interest)	Statistical count
		Little biological material necessary	Limited recovery–VBNC bacteria
		Non operator-dependent and rapidity of the preparation	
		No deadline for reading results (better recovery than HPC)	

after having been injured, but they can recover with time and thus stay potentially pathogenic.[28] This is of great concern when used in disinfection tests, since microorganisms undergo a stress situation even if they are not deactivated. Using this count procedure in disinfection tests includes these so-called ''viable but non culturable'' (VBNC) microorganisms into the bactericidal efficiency, while this loss of cultivability, actually due to oxidative stress, is not taken into account in reference blank tests.

We can distinguish three main variants in the HPC. The ''spread plate method'', used to numerate aerobic organisms in a sample. This procedure consists in spreading aliquots from serial dilutions (usually decimal) on the surface of a Petri dish containing an agar medium adapted to the growth of the bacteria. This can be done either by shaking glass beads onto the surface to disperse the inoculum or by using a glass pipette molten at its end to form a spreader. The main drawback of this method remains the operator dependency even if it is the most widely used due to its simplicity.

The ''pour plate method'' only differs that volumes of the sample or its dilutions are mixed with molten agar and incubated. Thus, most of the colonies are trapped in the agar, so that they remain small in size (Fig. 3). This spatially restricted growth avoids the merging of the colonies and thus facilitates the numeration. Furthermore, this allows Petri dishes to be positively incubated for a longer duration so that damaged cells can recover cultivability and form a colony. In addition, the use of warm molten agar can avoid some accidental contamination when working with heat resistant bacterial endospores by killing more sensitive contaminants. It is noteworthy that these two methods are adapted to concentrated samples (*i.e.* corresponding to low volume) since more than 200 μL cannot be absorbed by a standard 90 mm diameter agar plate. For larger volumes, *i.e.* weakly concentrated samples, the HPC method is adapted, so that volumes can be filtered through a 0.45 μm filter placed onto an adapted growth medium, leading to the membrane filtration count. Results are usually declared significant when the colony counts are between 25–250 colonies over an agar plate as recommended by the Food & Drugs Administration (FDA). This recommendation results from the fact that on the one side, a too low density would need too many replicates to give a meaningful result and on the other side, too high counts strongly increase the risk to enumerate colonies from a cluster or the fusion of colonies. The FDA also recommends that HPC computation should be expressed with only the first two significant digits to avoid creating a mistaken impression of accuracy.

Finally, the ''spiral plating method'' developed in the 70s is the only automation of HPC recommended by the FDA. This apparatus dispenses a decreasing volume of sample diluted in an increasing volume of solvent on the surface of a rotating

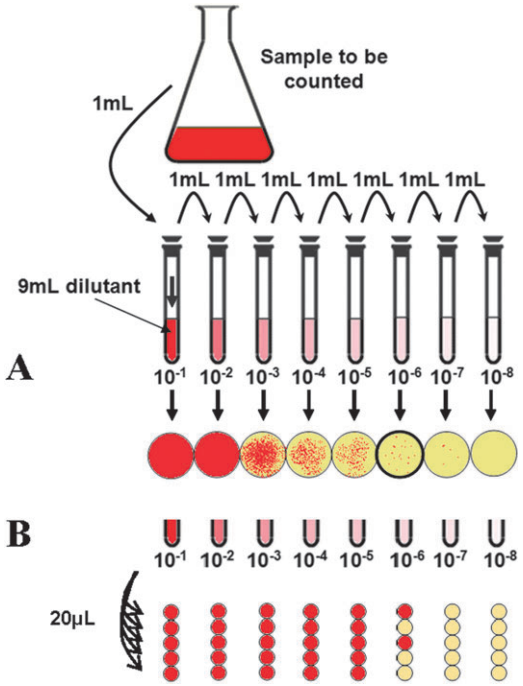

Fig. 2 Description of (A) the Heterotrophic Plate Count (HPC) and (B) the Most Probable Number (MPN) procedures. (A) The plate count procedure is used to count the cultivable population in a sample. Dilutions are achieved by adding an aliquot of the sample (*e.g.* 1 mL) to a sterile dilution tube containing dilutant (*e.g.* 9 mL). Greater dilutions are achieved by sequentially diluting the sample in series: adding 1 mL of the first dilution to 9 mL of sterile dilutant achieves an additional tenfold dilution. Spreading or pouring a known aliquot from each dilution to agar medium allows bacteria to form colonies. Only the plates showing counts in the range of 25–250 colonies are taken into account and the final concentration of CFU is obtained by multiplying with the appropriate dilution. (B) The MPN procedure follows at the initial stage that of HPC. Then, it involves inoculation (*e.g.* 20 µL) of multiple tubes with replicate samples of serial dilutions. The pattern of tubes that show growth (red tubes) is statistically related to the most probable number of cultivable bacteria in the sample.

Petri dish according to an Archimedes spiral pattern. The dispensed volume is calibrated and known at every point (*i.e.* in each dish sector) of the Petri dish so that the concentration is obtained by dividing the number of colonies enumerated by the volume dispensed in the same sector of the dish. However, this method requires expensive and very specific material so it is not commonly used.

5.2 Fluorescence techniques

For a few decades, fluorescence methods applied to biological counting have attracted a growing interest due to their easy-to-use nature.[29] Based on the fluorescent properties of dyes bound to specific cell components, fluorescence methods provide a direct count since they rely on the direct observation of the cells and allow targeting of very specific characteristics of the state or of the metabolic activity of a microorganism, like the membrane permeability, the respiration, the energy content,. . .. Hence, contrary to HPC, the results do not depend on the adequacy of the growth conditions and furthermore, many of the staining methods can be applied to all microorganisms. However, concerns remain as to the choice of an appropriate viability indicator when using such methods.

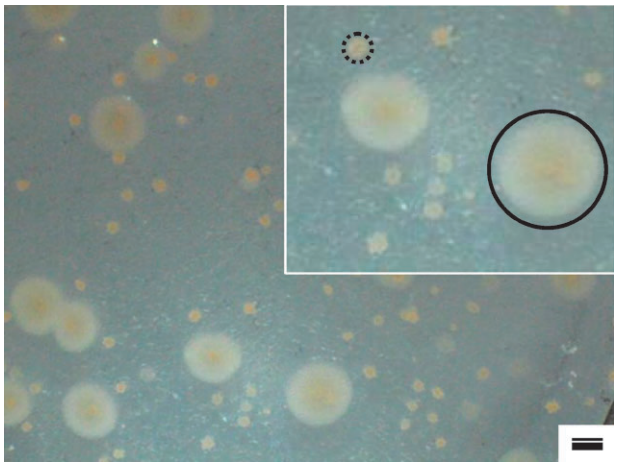

Fig. 3 Surface (full circle) and embedded (dashed circle) *B. subtilis* colonies on an LB agar plate by the "pour plate method". In contrast to surface colonies, the embedded or trapped colonies are limited in their expansion, and remain small even after a long incubation time. The scale bar corresponds to 1 mm (×10 image magnification).

The easiest way to count the number of viable bacteria is to perform fluorescence spectrometry, although higher precision results from the direct count of single cells with an epi-fluorescence microscope or a flow cytometer. *Epifluorescence* indicates that the excitation light passes through the objective, instead of through the sample, so that only the reflected excitation light has to be filtered from the emission light of the sample, thus giving a much higher intensity. The flow cytometer device lets a diluted sample flow through a capillary so that each cell remains separated from the others. Light sources, usually lasers, excite the stained cells and the forward scatter light (emitted in line with the source) as well as the side scatter emission are measured. Fine analysis can be obtained. Even if this valuable technique is a standard in many biological laboratories, it appears too sophisticated to be applied to routine photocatalytic disinfection tests.

The oldest staining method using fluorescence is the acridine orange (AO) direct count (Fig. 4A and B). Still commonly used, AO is a cell-permeable dye that strongly binds to nucleic acids. It is reported that it emits green light when bound to double stranded nucleic acid (DNA), while a red fluorescence is observed when associated to single stranded nucleic acid (RNA).[30] Thus, it was usually thought that metabolically active bacteria (those having high amounts of RNA, an intermediate needed in protein synthesis) were stained "red", whereas the dead ones were labelled "green". Since the AO direct count is not very often used nowadays, more details on its drawbacks and possible improvements can be found elsewhere.[31]

Kepner and Pratt have noticed that DAPI (4′,6-diamidino-2-phenylindole), a non-intercalating stain, is progressively replacing AO since the 80s:[29] this cell-permeable molecule strongly binds to DNA and then emits a bluish fluorescence

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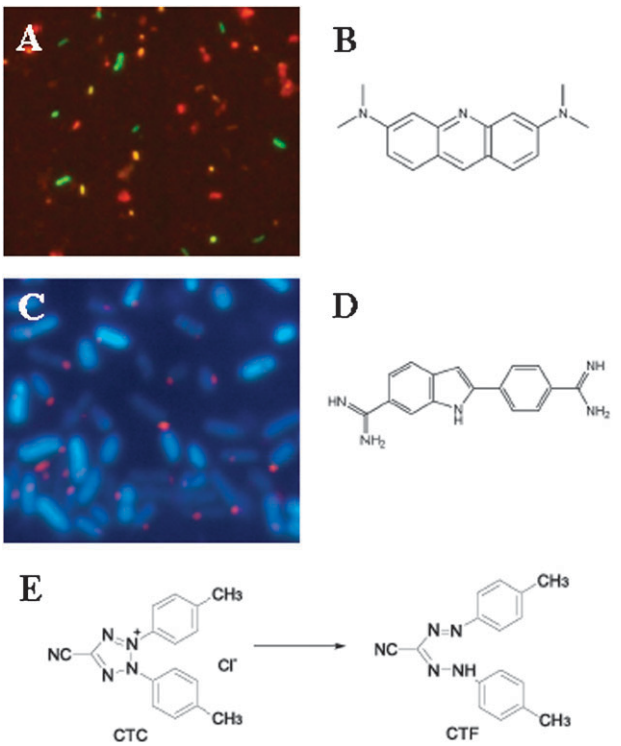


Fig. 4 (A) Single stain fluorescence technique with an epifluorescence image of *E. coli* stained with Acridine Orange—(B) The Acridine Orange molecule—(C) Double stain fluorescence technique, with an epifluorescence image of *E. coli* stained with DAPI-CTC. Cells with an active electron transfer chain (related to the respiration activity) reduce CTC to an insoluble formazan salt that emits red light—(D) Schemes of the DAPI and (E) CTC molecules, with the CTC reduction into CTF.

when excited under UV light (Fig. 4C–E). It can be used in combination with CTC (5-cyano-2,3-ditolyl tetrazolium chloride) which emits red light once reduced in the respiration chain of active bacteria (the blue fluorescence permits contrast with

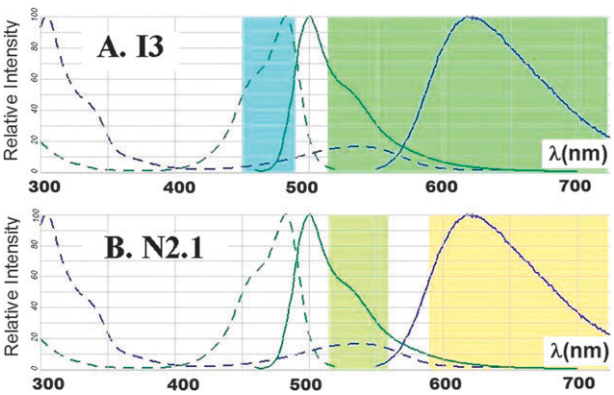
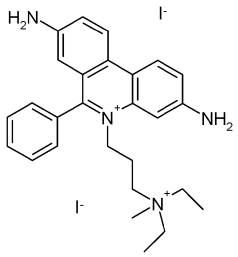


Fig. 5 (A) The 1st optical set "I3" (Leica, excitation filter: 450–490 nm, suppression filter > 515 nm) allows both live and dead cells to be seen due to the large excitation band of the PI–DNA complex (blue dashed line) which is partially included in that of the SYTO 9™–DNA complex (green dashed line). (B) The 2nd optical set "N2.1" (Leica, excitation filter: 515–560 nm, suppression filter >590 nm) excites only the PI–DNA complex (blue dashed line) and lets it fluoresce at its maximal emission wavelength (blue full line).



Scheme 2 Propidium iodide (PI).

the red emission). This allows at the same time a total count and a viable count including VBNC bacteria.[32] This set of dyes has been recently beneficially used in association with HPC to assess the decontamination efficiency of a patented UV-A photocatalytic reactor on an *E. coli* contaminated airflow.[6]

However, even if this DAPI-CTC double staining has been positively used for viability counts,[33] some concerns remain when used alone. Other metabolic tests yield much higher counts than CTC does and metabolically active bacteria cannot reduce CTC under some conditions.[34] Therefore, an increasing number of microbiologists prefer a double staining method based on a membrane integrity test "Baclight" (Invitrogen) which has proved its accuracy in many fields.[35] It is based on two stains, propidium iodide (PI) and SYTO9™, which differ in their ability to penetrate bacterial cells (Scheme 2).[36] This efficient numeration procedure is based on the non-selectivity of the SYTO9™ stain towards the integrity of cell membranes (Fig. 5): SYTO9™ penetrates the cell membrane whatever its integrity (integrate as well as damaged membranes), whereas PI only enters the damaged cells. With integrate membranes, the SYTO9™-DNA complex lets the living cells fluoresce green (excitation/emission maxima: 480 nm/500 nm), while the higher affinity of PI to DNA removes the SYTO9™-DNA complex and reacts with the DNA, which lets the dead cells emit red light (excitation/emission maxima: 530 nm/620 nm).

As a consequence, when used alone, SYTO9™ stain labels both living and dead bacteria, while in contrast, PI penetrates only bacteria with damaged membranes, reducing SYTO9™ fluorescence when both dyes are present. This membrane test is also particularly valuable for photocatalytic disinfection since it tests the part of the cell that is the most oxidized.[13]

This LIVE/DEAD BacLight Bacterial Viability kit was recently used to measure the efficiency of a TiO_2-based photocatalytic reactor towards bioaerosols contaminated with *L. pneumophila*.[6] The bacteria were collected downstream and filtered on a black Isopore Filter Membrane to concentrate the sample and then the filter obtained (filer staining method) was stained following the manufacturer's procedure. This method allows the preparation of an observable slide within about 20 min and was efficient on *Bacillus cereus*, *Bacillus subtilis* (*Bacillus atrophaeus*), *Clostridium perfringens*, *E. coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, *P. syringae*, *Salmonella oranienburg*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (manufacturer's indication).

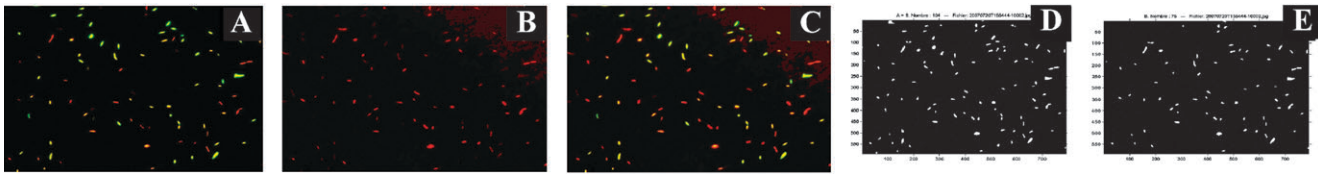


Fig. 6 Series of fluorescence images during the LIVE/DEAD BacLight Bacterial Viability kit BACLIGHT. However, the red fluorescence is not as pronounced as the SYTO 9™–DNA one (green full line) with the "I3" optical set so that we first add with Matlab the image (B) obtained with the "N2.1" to the image (A) set to enhance the contrast and thus place on the picture (C) all kind of cells. Then, Matlab treats those two images together and provides an estimation of the number of cells on each one (D–E). It is possible to get the orange cells erased from the picture if their colour is below a given value for the red component so that they then belong to the "green" label. Thus it identifies a class of bacteria for which the membrane state is "intermediate".

However, the apparent easiness of those methods hides a fundamental question that has to be answered when working with epifluorescence microscopy: when is the obtained result accurate? Kepner and Pratt reviewed over 200 articles and noticed many sources of variation such as the filter composition and porosity, the vacuum pressure of the filtration, the staining time.[29] The result accuracy depends mostly on the number of bacteria per microscope field, the number of observed fields and the number of filters prepared from an initial sample. They showed that most of the researchers count between 200 and about 400 cells displayed on 10 to 20 fields, but only on a single filter. At present, statistical considerations would rate counting fewer bacteria on fewer fields but on more replicate filters. It is clear that multiplying the number of filters increases the time needed for a count. Also, it should not be forgotten that counting less than 25 bacteria per field results in a coefficient of variation of 20% and that twice the number of bacteria per field decreases the variation coefficient of only 7%.[37] Hence, screening of photocatalysts with these methods renders this type of count rapidly fastidious for the operator whose attention could diminish and thus bias the results.

Direct counting using Baclight was carried out using Matlab 7.0 since this software allows easy image processing (Fig. 6).[6] This semi-automated image processing has many advantages compared to a "manual counting". First, since bacterial count is no longer time-consuming, one can snap microscope fields randomly without any time concerns and restrictions. This means, it is possible to discriminate particles that are either too large or too small, like dust, with some fixed rules. This prevents subjectivity of the operator deciding if a color patch is actually a bacterium or not.[29] Of course, an experienced eye would be sometimes more efficient but this would not be applicable when dealing with more than a thousand cells. It allows fixing of "color limits" to class bacteria between green and red labelled but also in an intermediate colour. Some emit orange light, actually the mix of green and red wavelengths, visualizing a more limited permeability of those membranes to PI due to a less damaged membrane. Using image processing also eliminates operator subjectivity and allows the possibility of recounting with the same results, which is, even with the same operator, sometimes difficult.

However, fluorescence microscopy suffers from drawbacks. First, even with automation for the image treatment, the preparation of many filters and the subsequent microscopic observations become fastidious if many analyses are to be performed in a given duration *e.g.* when following the abatement *vs.* time or during photocatalyst screening. Furthermore, with sediments like photocatalyst particles being filtered with the bacteria, the sample preparation becomes more difficult and time-consuming since it requires purification/separation extra-steps[38] which can bias the results. In the case of such interference, bacterial enumeration can quickly become impossible both by image processing and by eye (Fig. 7). A last point but not the least is the *quasi* absence of norms concerning direct count with fluorochromes. Only very few staining methods have been validated as standard methods and Kepner and Pratt noticed the lack of precision in the methodology used in the concerned articles.[29]

Another approach is based on the use of bacteria labelled with the Green Fluorescent Protein (GFP), which was first isolated from jelly fish *Aequorea victoria*.[39] The gene coding this protein can be introduced inside the genome of the bacteria. The expression of GFP in these genetically modified bacteria is a good indicator of the active metabolism of the cells and thus can be used to trace the impact of bactericidal treatments.

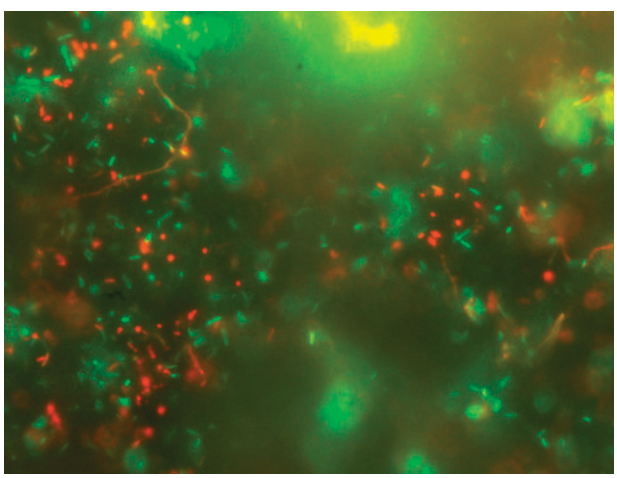


Fig. 7 Example of *L. pneumophila* stained with Baclight following manufacturer's procedure in presence of TiO_2 particles. The photocatalyst particles exhibit high fluorescence and cause severe problems in the depth of field (×1000 image magnification).

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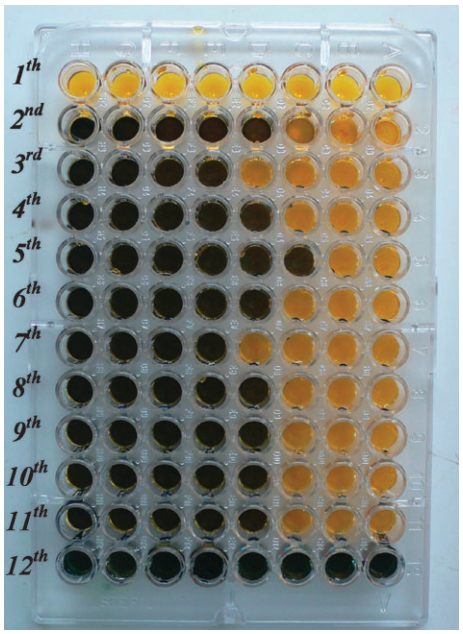

Fig. 8 Example of a microplate used to determine the MPN of *B. atrophaeus* in a sample. The first row is used for the ten-fold serial dilutions of the sample. The eleven following rows contain 30 μL LB agar medium implemented with soluble starch (2 g L^{-1}). Using a 8-channel micropipette, 20 μL of each diluted suspension is dispensed in the ten middle rows. The twelfth is inoculated with the dilution water to ensure sterility control. The plate is incubated for a few days at 37 °C to let the eventually damaged cells repair and multiply. Then 50 μL Lugol solution (yellow iodine containing solution) is placed with the multichannel pipette in all the wells to reveal growth: in the wells with bacteria multiplication, the starch is consumed so that the wells remain **yellow**, while in the wells where no growth occurred, the starch reacts with the iodine to a **deep purple complex**. The log(MPN) computed for this example is 5.2 with a 95% confidence interval 4.9–5.5, following the Excel spreadsheet procedure reported by Garthright and Blodgett.[40]

5.3 Most probable number methods (MPN)

The Most Probable Number method (MPN) is a procedure to estimate the population density of culturable microorganisms in a test sample. Aliquots from serial dilutions of a sample are inoculated into a liquid growth medium and incubated. The results of this process are, for each dilution, the number of tubes that display growth (Fig. 8). This pattern allows the statistical calculation of the MPN of culturable bacteria in the initial sample. The MPN estimation is based on the hypothesis that the organisms are randomly distributed throughout the liquid medium (*i.e.* there are no clusters), and that the incubated medium shows growth whenever the sample contains culturable organisms.

Our goal is not to provide statistical explanations of this method, already well-documented since its introduction in the middle of the 20th century and for which many computer programs exist.[40] We present basic principles to explain the calculation of the MPN in a sample. If there are k organisms in a volume of liquid V, and if a sub-sample of volume v is taken from V, the probability P that no organisms are in the sub-sample is:

$$P = (1 - v/V)^k$$

Hence, if n such sub-samples are taken, the probability $P_{s,n}$ that s of them will contain no organisms is:

$$P_{s,n} = C_n^s (P)^s (1 - P)^{n-s}$$

The MPN is the number of culturable organisms k_0 in the initial sample that maximizes the probability $P_{s,n}$ of obtaining the pattern "s sub-samples presenting no growth and $(n - s)$ showing bacterial multiplication". Better results are clearly obtained when inoculating many times n subsamples. This is done in few minutes with microplates (8 × 12 tubes) and a multichannel pipette.[41] An Excel spreadsheet is available online to compute the MPN in accordance with the FDA,[40] allowing calculations for various combinations of dilutions and replications, and confidence intervals.

The MPN method is claimed to be less precise than other methods,[42] even though it was positively tested many times *e.g.* for assessing the bactericidal activity of chemical disinfectants or for measuring *Listeria* contamination in poultry. MPN yields somewhat to higher counts than HPC so that on the log scale, disinfection efficiency may be lowered. However, one huge advantage is that it is not necessary to proceed rapidly to the numeration. The longer the plate is incubated, the more injured cells can recover and multiply. It would be improbable for bacteria stay in the VBNC state for a long time so that the omission of those cells should not be as critical as for HPC. This is valuable for photocatalytic disinfection especially when the microorganisms are submitted to oxidative stress. Then, by contrast to HPC or epifluorescence, the whole procedure, including the dilutions, is repeated. Usually, HPC count is done with replicates coming from a single serial dilution set of suspension as well as filters for the direct count. MPN is also non-sensitive to sediments, even if a bias can be induced if the cells form clusters with the particles. Finally, since the coloration pattern takes an unambiguous binary form, with either a yellow or a deep coloration depending on whether there is growth or not, there is absolutely no operator subjectivity in the MPN method and the results are obtained immediately. Thus the ease and rapidity of the method, the small amount of material used and the objectivity of the results make it a useful tool for photocatalyst screening. Additionally, all information relative to the count (start inoculum, dilution factor, replicates per dilution, medium, results) is available for publication, so that comparisons are easy.

5.4 Definition of the logarithmic reduction

The final point which we would like to emphasize is the widespread use of the "logarithmic reduction" (LR) when expressing results in microbiology: *e.g.* a disinfection efficiency will be reported in decimal logarithmic abatement, rather than in percent as when working with chemicals. Measuring bactericidal efficiencies, Vries and Hamilton noticed that no publication mentioned exactly how the "logarithmic reduction" was calculated, because two definitions coexist.[43] This is also true when the logarithmic reduction is used in photocatalytic applications.

The first way to compute this log-reduction, LR_1, also called "mean of the logs", needs to convert each count to its log value. Then, the mean of the logs for both control and test

experiments is calculated and the log reduction is the difference between these two values. The second possibility, LR_2, also called "log of the means", is the difference between the log values of the two means of the counts.

The authors explained that LR_1 may be preferred for statistical reasons, since this calculation moves data towards normality with a skewness coefficient close to zero. The second definition may be preferred because the efficiency of a process (after/before contamination ratio) can be directly calculated with $1–10^{-LR2}$. In a usual example, *e.g.* a bactericidal test using a chemical disinfectant,[43] the 1% relative difference between LR_1 and LR_2, obtained by estimating $(LR_1–LR_2)/LR_2$, becomes a 11% difference when comparing the fraction of organisms that survived, derived from $(10^{-LR1}–10^{-LR2})/10^{-LR1}$. An 11% difference might seem to be huge for researchers working in the chemistry field, but we should explain that such a difference remains fully within the error range in microbiology, for which a difference lower than a half-log is usually not considered as significant. Therefore, mentioning the way in which the logarithmic reduction has been calculated, although more elegant from a scientific point of view, is not an absolute necessity since the choice of the reduction method does not significantly alter the meaning of the obtained results.

6. Conclusions

The growing inter-disciplinarity occurring nowadays between numerous fields requires scientists to be continuously aware of *border-line* research, so that they can open the door to design new materials, new processes, to imagine new application fields, and in fact to make science a living and non-sclerosed matter. This contributes to set both science and scientists in phase with society and societal problems.

For a deep and profitable understanding of an inter-disciplinary field such as that between photocatalysis and life science, we have suggested that chemists have clearly to learn from microbiologists. We hope this review will help those researchers who would like to bridge the gap between chemistry, and photocatalysis especially, and life science.

The different numeration methods routinely used for evaluating the efficiency of photocatalytic action on microorganisms have been described and the relative heterotrophic plate counts, the fluorescence techniques by epifluorescence and cytometry, and the MPN method have been discussed. In term of standardization, it appears that the MPN method is a very adequate standardizable numeration method for an easy, cheap, and rapid evaluation of the biocidal efficiency level of photocatalysts and thus selecting efficient photocatalytic materials among binary or more complex ternary systems.

Research with biological agents as targets implies taking into account parameters that chemists are not used to considering. We have proposed that using bacterial spores as a representative and suitable tool is an elegant weapon to overcome the constraints introduced by the major differences between biological and chemical targets, and allows the restrictive requirements related to the use of living matter instead of chemicals to be met.

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