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How long can culturable bacteria and total DNA persist in environmental waters? The role of sunlight and solid particles

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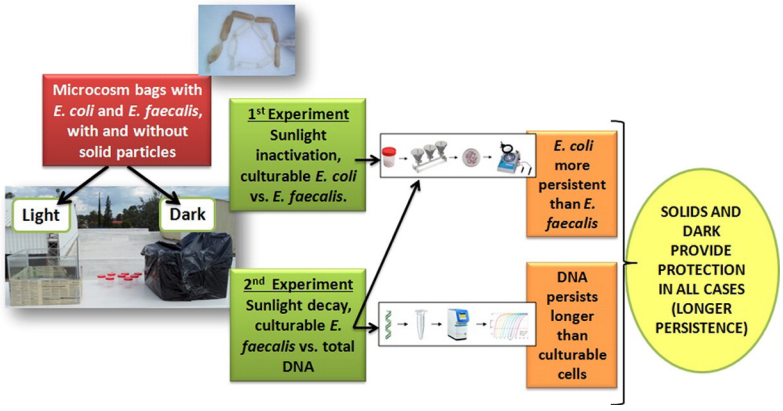
HIGHLIGHTS

- Sunlight inactivation of bacteria interacting with solid particles was assessed.
- Indicator bacteria without particles exposed to sunlight suffered immediate decay.
- Solid particles protected bacteria and DNA from sunlight effect.
- Culturable *E. coli* was more persistent than *E. faecalis* in all conditions evaluated.
- In all cases analyzed, DNA from *E. faecalis* persisted longer than culturable cells.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, sunlight inactivation of two indicator bacteria in freshwater, with and without solid particles, was studied and the persistence of culturable cells and total DNA was compared. Environmental water was used to prepare two matrices, with and without solid particles, which were spiked with *Escherichia coli* and *Enterococcus faecalis*. These matrices were used to prepare microcosm bags that were placed in two containers: one exposed to sunlight and the other in the dark. During one month, samples were removed from each container and detection was done by membrane filter technique and real-time PCR. Kinetic parameters were calculated to assess sunlight effect. Indicator bacteria without solid particles exposed to sunlight suffered an immediate decay (<4 h) compared with the ones which were shielded from them. In addition, the survival of both bacteria with solid particles varied depending on the situation analyzed (T_{99} from 3 up to 60 days), being always culturable *E. coli* more persistent than *E. faecalis*. On the other side, *E. faecalis* DNA persisted much longer than culturable cells (T_{99} > 40 h in the dark with particles). In this case active cells were more prone to sunlight than total DNA and the protective effect of solid particles was also observed. Results highlight that the effects caused by the parameters which describe the behavior of culturable microorganisms and total DNA in water are different and must be included in simulation models but without forgetting that these parameters will also depend on bacterial properties, sensitizers, composition, type, and uses of the aquatic environment under assessment.

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1. Introduction

Inactivation by sunlight is one of the main abiotic factors that affect the persistence and survival of microorganisms in aquatic environments (Bae and Wuertz, 2009). It is widely used as a process for water disinfection (Boyle et al., 2008; Shannon et al., 2008; Wegelin et al., 1994) and has demonstrated an important role in improving water quality mainly in places with warm and dry weather (Salih, 2003). However, this treatment may not lead to the total removal of microorganisms due to different factors. Inactivation rate depends, for example, on the microorganism position in the aquatic environment and it is different when located in the surface, in the middle, or in the bottom of the water column (Rozen and Belkin, 2001). On the other hand, it is not clear whether the effect of inactivation by sunlight produces cell lysis or if it causes that microorganisms enter in an active but non-culturable state, and moreover if these cells preserve their infectivity (Smith et al., 2000). In addition, the presence of suspended particulate matter may interfere with the inactivation of microorganisms by sunlight (Boehm and Sassoubre, 2014; Brauwere et al., 2014; Rozen and Belkin, 2001), giving them protection and allowing their survival for longer periods of time. Intense rainfall and also recreational activities may increase the bed shear stress producing sediment resuspension (Walters et al., 2014a) in several aquatic environments, thus increasing water turbidity (Gutiérrez-Cacciabue et al., 2014). Microorganisms present in water may be adsorbed to these particles and can be transported with the stream reaching further locations or being deposited together with the sediments onto the river bed, contributing to the already existing microbial reservoir (Gao et al., 2011). Both fates have an impact on the health risk of recreational users due to an eventual ingestion (Chandran et al., 2011), especially when the conditions (high turbulence) are such that microorganisms are desorbed, returning to the water column (Kay et al., 2005).

Due to the difficulty of direct measuring each of the pathogens that can be present in water, the use of fecal indicator bacteria (FIB) has been established by legislation. *Escherichia coli* and enterococci are within the FIB group that is commonly used to assess the quality of recreational waters. These microorganisms are usually detected by culture-based methods, which are easy to perform and involve low costs (Ashbolt et al., 2001). The limitations of these techniques are that the results are obtained within 24–48 h and allow the detection of culturable cells, but do not allow to detect damaged or starving cells or cells that are active but not culturable or non-viable cells (Oliver, 2005; Salam, 2011), underestimating the true number of microorganisms and the real health risk. To avoid this problem, the use of culture-independent methods, such as quantitative polymerase chain reaction (qPCR), has been proposed (Agudelo et al., 2010; Duris et al., 2009; Haugland et al., 2005; Poma et al., 2012a; Rajal et al., 2007). However, this technique allows the detection of total DNA but cannot distinguish between viable and non-viable cells due to the persistence of DNA in the environment after the cell has lost its viability (Salam, 2011), overestimating or even providing false positive results in the absence of viable cells (Kell et al., 1998). Regarding costs, those for qPCR are higher than for culture-based methods, which especially have an impact on developing countries, where resources for equipment and reagents are limited.

Even though the Environmental Protection Agency of the United States (Method A, EPA, 2010) has presented a report in order to include the use of molecular techniques to assess enterococci in recreational waters, there are still concerns to be solved with respect to the fate and transport of microorganisms in water and the real health risk related to this situation.

Therefore, the aims of this work were: a) to study the effect of sunlight on the survival of two bacterial indicators, *E. coli* and enterococci, with and without the presence of solid particles, and b) to measure enterococci by two different methodologies: traditional microbiological methods and molecular techniques (qPCR) to compare the persistence of culturable enterococci and total DNA when exposed (or not) to sunlight also with and without solid particles.

2. Materials and methods

Two experiments were carried out in order to assess the inactivation of culturable microorganisms and the decay of total DNA by sunlight, evaluating the influence of solid particles. In the first experiment, the inactivation of two bacterial indicators widely used for water quality assessment, *E. coli* (selected as a model of Gram-negative bacteria) and *Enterococcus faecalis* (as a model of Gram-positive), was compared quantifying them by culture based methods; and in the second one, a comparison of the persistence of culturable enterococci and its total DNA when exposed to sunlight, with and without solid particles, was made by using two different methods for their enumeration: a culture-based and a culture-independent one.

2.1. Sample collection

Water and sediment were collected from aquatic environments that belong to the Province of Salta, Argentina (Gutiérrez-Cacciabue et al., 2014). Sediments were dried, classified by size, and kept in black bags in the laboratory, before use. Ten liters of environmental water were collected in clean plastic containers following the *Standard Method for Examination of Water and Wastewater for Surface Waters* (Eaton et al., 2005). Plastic containers were rinsed with the environmental water before filling them. Water samples were taken to the laboratory and immediately used (within 1 h after collection) for matrices preparation. Both solid particles and environmental water were not sterilized for the experiment in order to keep the natural flora intact and thus to allow the interaction between them and bacteria used.

To identify initial water condition, physicochemical parameters such as temperature (T, in °C), dissolved oxygen (DO, in mg/l), turbidity (TURB, in NTU), conductivity (COND, in μS/cm), and pH were measured in situ using a multiparametric analyzer U10 (Horiba, Japan), and microbiological assays (*E. coli* and enterococci) were performed at the laboratory by membrane filter technique (Eaton et al., 2005).

For the first experiment water from Vaqueros River was collected for matrix preparation and for the second one, water from La Caldera River was used (due to the lack of water in Vaqueros River). Regarding sediments, the same were used for both experiments.

2.2. Inocula preparation

ATCC strains were used to prepare stock solutions of two bacteria: *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212); the last one was chosen as a representative of the enterococci group. This procedure consisted briefly in incubating each strain in nutrient broth (Britania, Argentina) at 37 °C for 18 h. After that, the broth was centrifuged (10 min 4000 ×*g*). The supernatant was discarded, the pellet was washed twice with phosphate buffered saline (PBS) 1×, and finally each stock solution was obtained by resuspending the cells in PBS.

For the first experiment the stock concentration was around 10^6 CFU/ml for both strains and for the second one, the stock was approximately 10^9 CFU/ml just for *E. faecalis*. This higher concentration used in the second experiment, in which enterococci were enumerated by two different methods, was selected to reach qPCR detection limits.

2.3. Matrix preparation

Sterilized glass bottles (2 l) were used to prepare four matrices; each of them was filled with 2 l of the collected environmental water. In two of the four bottles, 10 g of solid particles with diameter <44 μm were added (final concentration: 5 g/l). The concentration of total solids chosen corresponded to a situation of maximum turbidity found (>900 NTU) in some of the aquatic environments previously monitored

(Gutiérrez-Cacciabue et al., 2014). In the other two bottles, no solid particles were added (simulating clear water, TURB <3 NTU). Both, the sediment concentration (5 g/l) and the size (<44 μm) selected for this experiment, were considered as the most favorable for microorganisms survival.

2.4. Experiment setup

In the first experiment, microcosm bags were employed to assess the inactivation rates of *E. coli* and *E. faecalis* in water by sunlight. For that, each of the four water matrices prepared, was inoculated with 300 μl of the stock of the selected indicators bacteria (to reach a final concentration in the order of 10^3 CFU/ml): two matrices (one with and one without solid particles) with *E. coli* and the other two (one with and one without solid particles) with *E. faecalis*. All matrices were properly mixed to allow a uniform distribution of microorganisms and their interaction with particles.

For microcosm bags design a cellulose dialysis tubing (MWCO 12400 Da, Sigma-Aldrich, USA), previously treated following manufacturer instructions (Sigma-Aldrich, USA), was cut into equal 10 or 20 cm-length pieces. These "bags" were filled separately with the four different water matrices and tied at the ends, representing microcosms that simulate the behavior of bacteria in an aquatic environment (Bae and Wuertz, 2009) considering a "one time" contamination event. Microcosm bags with *E. coli* and *E. faecalis*, with and without solid particles, were divided in equal amounts and deposited in two glass containers (50 × 30 × 40 cm) that were previously filled with 20 l of the same environmental water used to prepare them (river water). One of the containers was exposed to sunlight while the other was covered with black bags to avoid sunlight exposure (Fig. 1a). In addition, all the sides of the containers were wrapped with dark paper to avoid the side effects of solar radiation. For the first experiment, both containers were divided in two parts to distinguish between bags with *E. faecalis* from the ones spiked with *E. coli*. To obtain results as close as possible to those under real environmental conditions, experiment was performed outdoor. Thus, the microcosm bags could be exposed to other factors such as rainfall and changes in temperature between day and night. Also, it was possible to favor the interaction with specific characteristics of the environmental water used such as dissolved organic matter, solids and predators (Bae and Wuertz, 2009). That was the reason for not sterilizing the environmental sediments or the river water.

During one month, microcosm bags with *E. coli* and *E. faecalis*, with and without solid particles, were removed in duplicates from each container, exposed to light and in the dark (16 bags in total), and taken to the laboratory for microbiological analysis. In the first experiment, only culturable bacteria were measured by membrane filter technique (Eaton et al., 2005) (Fig. 1a).

In addition, three physicochemical variables (pH, T, and DO) of the water in both containers (dark and light) were monitored at each sampling time using the multiparametric analyzer (U10, Horiba, Japan) to detect any possible change. Solar radiation data measured at every hour during all the experiment was provided by the Research Institute of Non-Conventional Energy (INENCO, CONICET — UNSa), University of Salta, Argentina. Environmental water in both glass containers was totally renewed every three days to avoid the decrease on the level of water by evaporation (mainly in the container exposed to sunlight) and also to avoid water quality changes due to the stagnation. Permanent aeration was maintained through aquarium aerators.

A second experiment was performed following the same procedure mentioned before but in this case two glass bottles with 2 l of environmental water each, one with and the other without solid particles, were inoculated with 2 ml of the stock of *E. faecalis* (to reach a final concentration of 10^6 CFU/ml). These water matrices were used to prepare the microcosm bags. Then, the bags were placed in the containers, one exposed to sunlight and the other shielded from it. As in the first experiment, during one month microcosm bags with and without solids, in duplicate, were removed from both containers (8 bags in total) for microbiological analysis (Fig. 1b). *E. faecalis* quantification was carried out by membrane filter technique (Eaton et al., 2005) and qPCR (Frahm and Obst, 2003) (Fig. 1b). Physicochemical variables (pH, T, and DO) from both containers were also measured at each sampling time.

2.5. Enumeration of culturable E. coli and E. faecalis

Culturable *E. faecalis* and *E. coli* were quantified by membrane filter technique (Eaton et al., 2005). *E. coli* was incubated in modified mTEC Agar (Fluka, USA) at 44.5 °C for 24 h (Method 1603, EPA, 2002a), and *E. faecalis* in mE Agar (Difco, USA) at 41 °C for 48 h and for confirmation in Esculin-Iron Agar (EIA) at 41 °C for 20 min (Method 1106.1, EPA, 2002b).

2.6. DNA extraction and detection by qPCR

Due to the existence of inhibition in most environmental samples (Poma et al., 2012b; Rajal et al., 2007; Schriewer et al., 2011), it was necessary to perform a nucleic acids extraction-purification step prior to qPCR. In this case, enterococci DNA was extracted from 200 μl of the sample using a commercial kit Pure Link™ Viral RNA/DNA (Invitrogen User Manual, USA, 2006). The procedure was performed following the manufacturer instructions.

Taqman qPCR reactions were carried out in duplicate in a GeneAmp®5700 Sequence Detection System (Applied Biosystems). Primers and probe used for enterococci detection were previously designed and validated (Frahm and Obst, 2003) and their concentration was optimized (data not shown). The combination of oligonucleotide concentrations that provided better amplification curves were 900 nM for both primers and 250 nM for the probe. Each 25 μl qPCR amplification reaction mixture contained 12.5 μl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 5 μl of extracted DNA, optimized concentration of primers and probe and nanopure water to complete the reaction volume. Negative controls were run in the same way but in this case nanopure water was used instead of DNA sample. Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C (Frahm and Obst, 2003) A serial dilution approach was employed for each sample in order to mitigate inhibitors during qPCR amplification (Poma et al., 2012a, 2012b; Rajal et al., 2007; Schriewer et al., 2011). Results obtained were analyzed with the GeneAmp®5700 SDS software.

The standard curve was built using a 1/10 dilution series with DNA extracted from a pure culture of *E. faecalis* (1×10^{10} CFU/ml), previously growth in nutrient broth. The threshold cycles (C_t) were calculated using baseline values of 6 to 15 and a threshold of 0.02. The enterococci concentration in each sample was calculated from each C_t obtained from the qPCR reactions.

2.7. Data analysis

Inactivation rates were calculated following a first order decay model according to Chick Law (Chick, 1910):

$$\frac{C}{C_0} = e^{-k_{si}t} \quad (1)$$

where C_0 and C are the initial bacteria concentration and bacteria concentration over time t (h) in CFU/100 ml, respectively, and k_{si} (h^{-1}) is the inactivation constant rate, which was calculated as the slope of the linear regression from the plot of the logarithm of the concentration ratio (C/C_0) vs. time.

In addition, the coefficient of determination (R^2) was obtained from the regression analysis. It is a statistical value that measures the degree of linear association between x and y-axis, indicating how well the

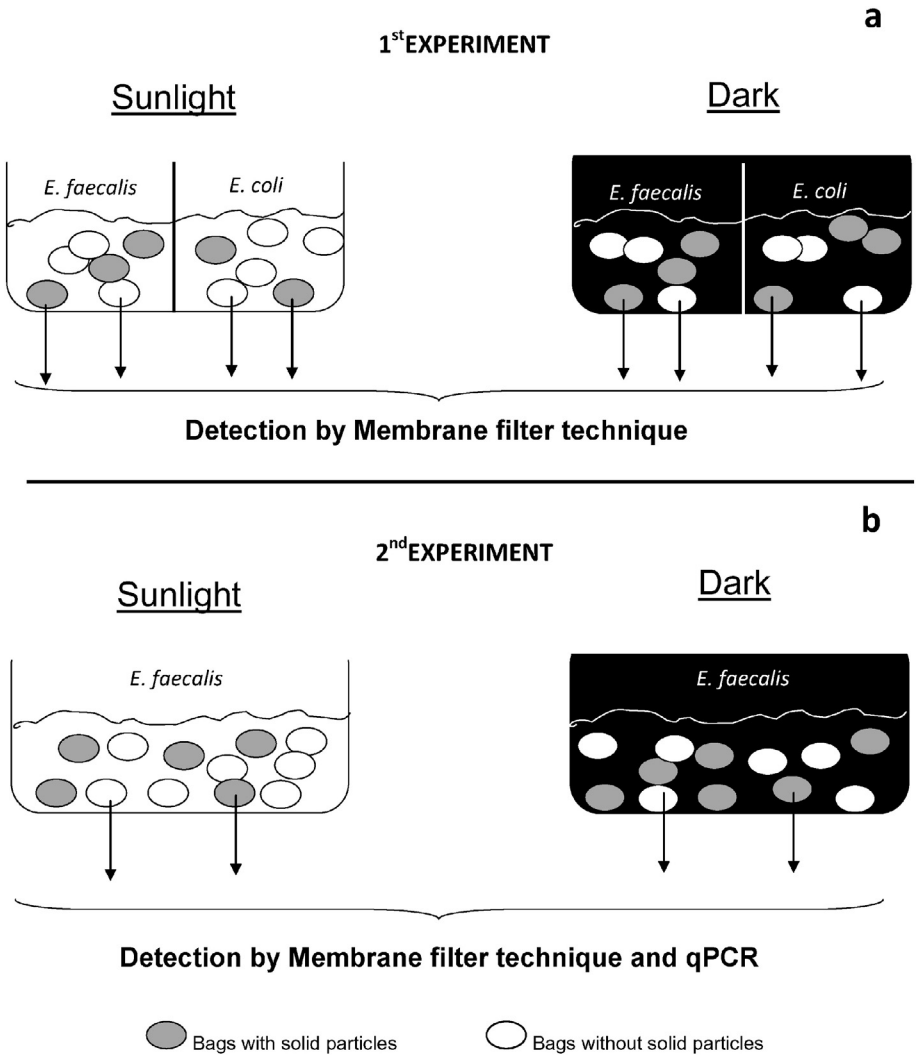

Fig. 1. Experiment setup: 1st Experiment: Sunlight inactivation of culturable *E. coli* and *E. faecalis*, (b) 2nd Experiment: Sunlight inactivation of culturable *E. faecalis* and decay of its total DNA, both experiments with and without solid particles. White recipients contain microcosm bags exposed to sunlight and the black ones hold microcosm bags in the dark.

regression equation fits the sample data. The time required for the decay of the 99% of the microorganisms (T_{99}) was calculated as:

$$T_{99} = \frac{\mathrm{Ln}\,(100)}{k_{si}}. \quad (2)$$

The time for a population to reach a half reduction ($t_{1/2}$) was calculated as:

$$t_{1/2} = \frac{Ln(2)}{k_{si}}. \quad (3)$$

Statistical analyses were performed using the statistical software package InfoStat (Di Rienzo et al., 2011). Due to the non-parametric distribution of the data ($p < 0.001$), the Kruskal–Wallis technique was used to assess significant differences between groups of variables. Differences were considered significant when p values were less than 0.05.

3. Results and discussion

3.1. Initial water quality and environmental conditions

The first experiment was conducted in early 2011 at the end of wet season (March–April). The average rainfall during these periods were 78.5 mm and 41.1 mm, respectively (INTA/EEA, 2012). Radiation values ranged from 0 to 800 W/m^2 (data not shown, provided by INENCO, CONICET — UNSa).

Water used for microcosm bags preparation, which was collected from Vaqueros River (Gutiérrez-Cacciabue et al., 2014), presented the following characteristics: T: 18.8 °C; pH: 7.93; DO 10.04 mg/l; COND: 84 μS/cm; TURB: 3 NTU; *E. coli*: 41 CFU/100 ml, and enterococci: 50 CFU/100 ml.

Some physicochemical values (DO, pH, and T) were also monitored at each sampling time in the water of both containers (Table 1). Results indicated that these parameters did not substantially change during the sampling time (Table 1) and also the differences between dark and night were not significant for neither of the parameters measured ($p > 0.05$).

The second experiment was carried out at the end of dry season in late 2012 (November). The average rainfall for this month was 29.7 mm (INTA/EEA, 2012). Radiation values ranged from 0 to 1000 W/m^2 (INENCO–CONICET, UNSa). In this case, water from La Caldera River (Gutiérrez-Cacciabue et al., 2014) was collected to prepare the microcosm bags. Physicochemical and microbiological parameters of the water initially measured were: T: 20 °C; pH: 9.00; DO: 7.00 mg/l; COND: 160 μS/cm, and TURB: 11 NTU, *E. coli*: not detected in 100 ml and enterococci: 26 CFU/100 ml. As in the first experiment, physicochemical variables measured at each sampling time in the

water of both containers did not significantly change (Table 1). In addition, the different physicochemical values found in both containers were not significant ($p > 0.05$).

It is important to highlight that the dissimilarity observed between the physicochemical values measured in both experiments ($p < 0.05$) were expected and may be explained to the fact that the water used for matrices preparation and also the period of time at which each experiment was carried out, were not the same (precipitation regime, lack of water in some of the rivers, ambient temperature). Although the aim of this study was not to compare bacteria behavior between different water matrices when exposed to sunlight, it is important to keep in mind that water type (freshwater from different origins, seawater or spring waters) and environmental conditions (season time, rainwater) may also have an influence on bacteria persistence and survival and therefore, in the kinetic parameters obtained (Ferguson et al., 2003). Bae and Wuertz (2015) stated that it is not easy to compare decay kinetics constants for genetic markers in environmental waters when they have been determinate at different temperatures and water type, and they found that *Bacteroidales* decay constants were a function of temperature and salinity.

On the other side, Gutiérrez-Cacciabue et al. (2014) found similarities between La Caldera and Vaqueros Rivers in terms of physicochemical and microbiological characteristics, using multivariate techniques, mainly due to their geographical proximity and recreational uses. This suggests that differences between both matrices were not as relevant as one might think.

3.2. Sunlight inactivation of culturable *E. coli* and *E. faecalis*

The effect of sunlight and the presence of solid particles on the behavior of *E. coli* and *E. faecalis* was assessed by comparing inactivation constants rates, half-reduction times, and T_{99} of the bacteria incubated in different microcosm bags, exposed to sunlight or in the dark, with and without solid particles (Table 2, Fig. 2).

E. coli in the absence of solid particles and *E. faecalis* with and without them, exposed to sunlight, suffered an immediate inactivation (3 log reduction) in less than four hours (Fig. 2a, c). Therefore, it was impossible to calculate inactivation kinetic parameters. This may indicate that not only sunlight has an effect on microorganism's survival but also other environmental or water conditions (Korajkic et al., 2013; Manjit, 2002) may contribute to that.

Since water and sediments were not sterilized, indicator survival may have been affected by other factors such as the interaction with the ubiquitous microbiota present in the environmental water and sediments. These interactions could be neutral or produce detrimental or beneficial effect on one or both of the species involved. Autochthonous microbiota may have a negative effect on microorganisms survival attributed also to the advantage when competing for nutrients in the water. Introduced microorganisms need a previous time of adaptation to the environment to eventually persist or grow (Adesioye and Ogunjobi, 2013). In addition, the presence of predators can be another reason for the rapid bacteria decay in water (Domínguez et al., 2012; Manjit, 2002; McCambridge and McMeekin, 1981).

The persistence of *E. coli* in the dark was higher than when exposed to sunlight both with and without solid particles (Fig. 2a, b). In the dark, inactivation constant rate for *E. coli* without solid particles was near 6 times greater than in the presence of them (Table 2). Also, the time required to reduce 99% of *E. coli* in the dark was higher both with and without solid particles compared with *E. coli* with solid particles exposed to sunlight (Table 2). Results showed that sunlight had a remarkable effect on *E. coli* inactivation and this was more harmful in the absence of solid particles (Fig. 2a, b). Our results agree with Burton et al. (1987) who found that the survival of *E. coli* in sediments was higher than in their absence with more than 25% content of clays in their composition, due to the high concentration of organic matter.

On the other hand, *E. faecalis* persisted longer in the dark than in sunlight (Fig. 2d). Although it was observed that *E. faecalis* suffered less inactivation in the dark than in the light (Fig. 2c, d), with and without solid particles, the time needed for this inactivation in both cases was lower compared to that for *E. coli* exposed to sunlight with solid particles (Table 2). In addition, in the dark with solid particles, which represent the best situation for microorganisms survival, *E. coli* ($k_{si} = 0.0034\ h^{-1}$) was almost 32 times more persistent than *E. faecalis* ($k_{si} = 0.1\ h^{-1}$) in the same situation.

These results are contrary to those from other researchers who found smaller inactivation constant rates for enterococci (greater persistence), but in that case the experiments were performed with seawater (Bae and Wuertz, 2009; Boehm et al., 2005; Rozen and Belkin, 2001). Gin and Goh (2013) stated that enterococci may adapt better and survive longer in seawater than in freshwater. Bacterial wall structure may be another reason for this behavior. Gram-positive bacteria are more sensitive to environmental stressors than Gram-negative, due to the absence of the outer membrane. This membrane, that is only present in Gram negative bacteria, confers resistance and may provide protection from adverse environmental conditions (Silhavy et al., 2010).

Solid particles caused a significant decrease in inactivation rates (Table 2) acting as shields and preventing the penetration of sunlight into the cells (Alkan et al., 1995). Several aquatic environments that belong to the province of Salta, northwest Argentina, are widely used for recreational purposes, standing out primary contact activities in summer (Gutiérrez-Cacciabue et al., 2014). In this area, rainfall occurs during summer (wet season) and increases the flow rate and turbulence of rivers, resuspending small particles like clays that enhance water turbidity (Baudino, 1997). Also, bathers can be responsible for particle resuspension. These particles may protect microorganisms attached to them, acting as a barrier for sunlight inactivation. In addition, solid particles not only protect microorganisms from predators and other environmental stressors (Walters et al., 2014b), but they also provide benefits such as access to nutrients. As a consequence, microorganisms survive for longer periods in water and, after resuspension they can be transported to other parts of the environment (Boehm and Sassoubre, 2014; Walters et al., 2014b; Brauwere et al., 2014). All of this can increase the risk of getting a waterborne disease (Chandran et al., 2011).

The problem of microorganisms attached to particles which can eventually settle down and persist in the bed of the aquatic environment lies in the fact that during systematic water monitoring in the dry season (in the absence of bathers and low flow rate), false negatives

Table 1
Average values of the physicochemical parameters of the water in the two containers (light and dark) measured at each sampling time for 1st and 2nd experiment.

	1st experiment		2nd experiment	
	In the light	In the dark	In the light	In the dark
Temperature (°C)	24.2 ± 3.25	23.9 ± 4.08	29.4 ± 3.98	27.5 ± 3.13
pH	8.2 ± 0.22	8.0 ± 0.24	8.6 ± 0.39	8.5 ± 0.39
Dissolved oxygen (mg/l)	7.8 ± 1.35	7.5 ± 1.52	6.4 ± 0.85	6.7 ± 0.69

Table 2
Kinetic parameters for the inactivation of culturable *E. coli* and *E. faecalis* exposed to sunlight and in the dark, with (w/s) and without (wo/s) solid particles.

Condition	Bacteria	Solids	k_{si} (h^{-1})	$t_{1/2}$ (h)	T_{99} (h)	R^2
Sunlight	*E. coli*	wo/s	–	–	–	–
		w/s	0.0652	10.6	70.6	0.9403
	E. faecalis	wo/s	–	–	–	–
		w/s	–	–	–	–
Dark	*E. coli*	wo/s	0.021	33.0	219.3	0.8239
		w/s	0.0034	203.9	1354.5	0.8087
	E. faecalis	wo/s	0.11	6.3	41.9	0.9835
		w/s	0.10	6.9	45.6	0.8786

k_{si}: inactivation kinetic constant, $t_{1/2}$: half-reduction time (time for 50% reduction), T_{99}: time for 2 log reduction, R^2: coefficient of determination.

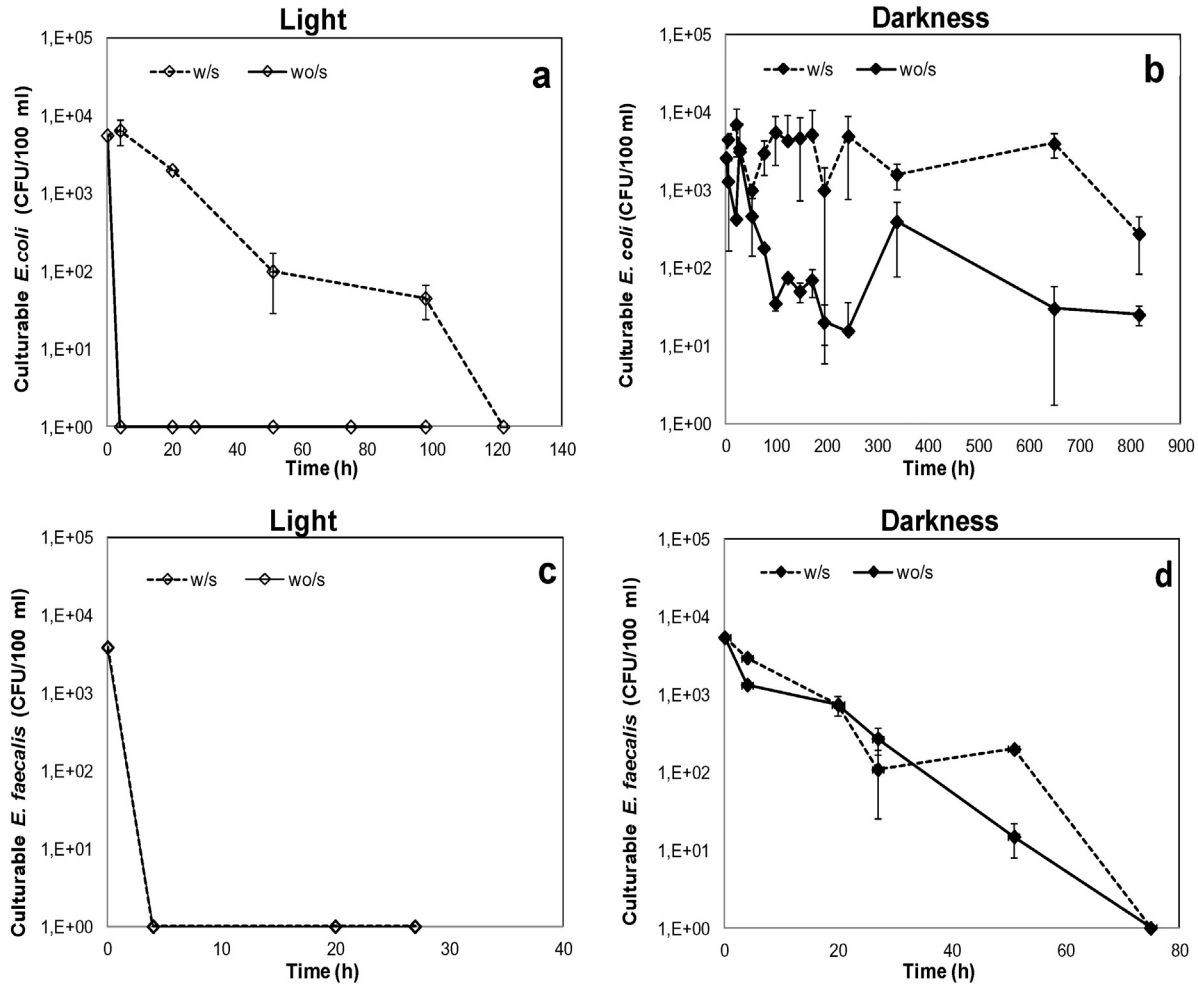

Fig. 2. Decay curves of culturable indicator bacteria *E. coli* (a, b) and *E. faecalis* (c, d) exposed to sunlight (a, c) and in the dark (b, d), with (w/s) and without (wo/s) solid particles.

can be obtained. This may be because, for microorganism quantification, samples are usually grabbed from the water surface (water with low turbidity), leaving on side microorganisms located at the bottom of the aquatic environment. However, during the wet season when a resuspension and an eventual desorption of microorganisms from the particles occurs, some of them return to the surface and can be detected. These results will have direct influence on quantitative microbial risk assessment (QMRA) because the risk of getting a waterborne disease will vary depending on the monitoring period (dry or wet season). That is why it is important to carry out systematic and seasonal monitoring (Gutiérrez-Cacciabue et al., 2014), considering that during the wet season particle resuspension will return microorganisms back to the surface, changing the concentration and eventually the risk estimation. Detection of these changes will allow reaching better results and addressing the risk from a more realistic perspective.

It is well documented that sunlight combined with oxygen, pH, and the presence of exogenous sensitizers such as humic substances can accelerate the photo-oxidative damage by producing destructive agents such as free radicals (O_2^-) and hydrogen peroxide (Curtis et al., 1992; Kadir and Nelson, 2014; Reed et al., 2000); therefore, authors suggested that these variables must be considered in future simulation models. Kadir and Nelson (2014) found that *E. coli* inactivation in pond water depended on dissolved oxygen concentration and UVB light. However, it was not as susceptible to exogenous mechanisms as *E. faecalis*, whose inactivation rate was 7 times greater than that of *E. coli*. In another study, Adesioye and Ogunjobi (2013) concluded that the long persistence of *Shigella* exposed to sunlight (up to 56 days) could be the result of the presence of high total dissolved solids and total organic carbon.

Finally, some authors had found a greater persistence of different pathogens such as *E. coli* 0157:H7 (Jenkins et al., 2011) and *Shigella* and *Salmonella* (Adesioye and Ogunjobi, 2013) over fecal indicator bacteria in water exposed to sunlight. Also, viruses like adenoviruses and some protozoa (*Acanthamoeba*) are highly UV resistant (Hijnen et al., 2006). This indicates that persistence and survival of microorganisms can differ between them and it is not quite correct to use results from one microorganism to assess the behavior of another.

3.3. Sunlight inactivation of culturable E. faecalis and decay of total enterococci DNA

In the second experiment qPCR (culture-independent method) was used to assess the persistence of total enterococci DNA. This technique has greater sensitivity and specificity, it is faster, and also provides information of the total population (Keer and Birch, 2003) including viable, dead, and damaged cells, and also extracellular DNA (Bae and Wuertz, 2009). Results from qPCR (total DNA) were compared with culturable *E. faecalis* enumerated by the membrane filtration method.

It is important to highlight that in the first experiment it was not possible to calculate kinetic constants rates for *E. faecalis* because of the loss of culturability almost immediately after the beginning of the experiment (Fig. 2c). Therefore, in this second experiment, the first sample was collected at a time closer to the beginning of experiment to meet the first part of the curve and to be able to do the linear regression (Fig. 3a). Besides, the concentration of the initial stock was higher, to ensure working over the qPCR detection limit.

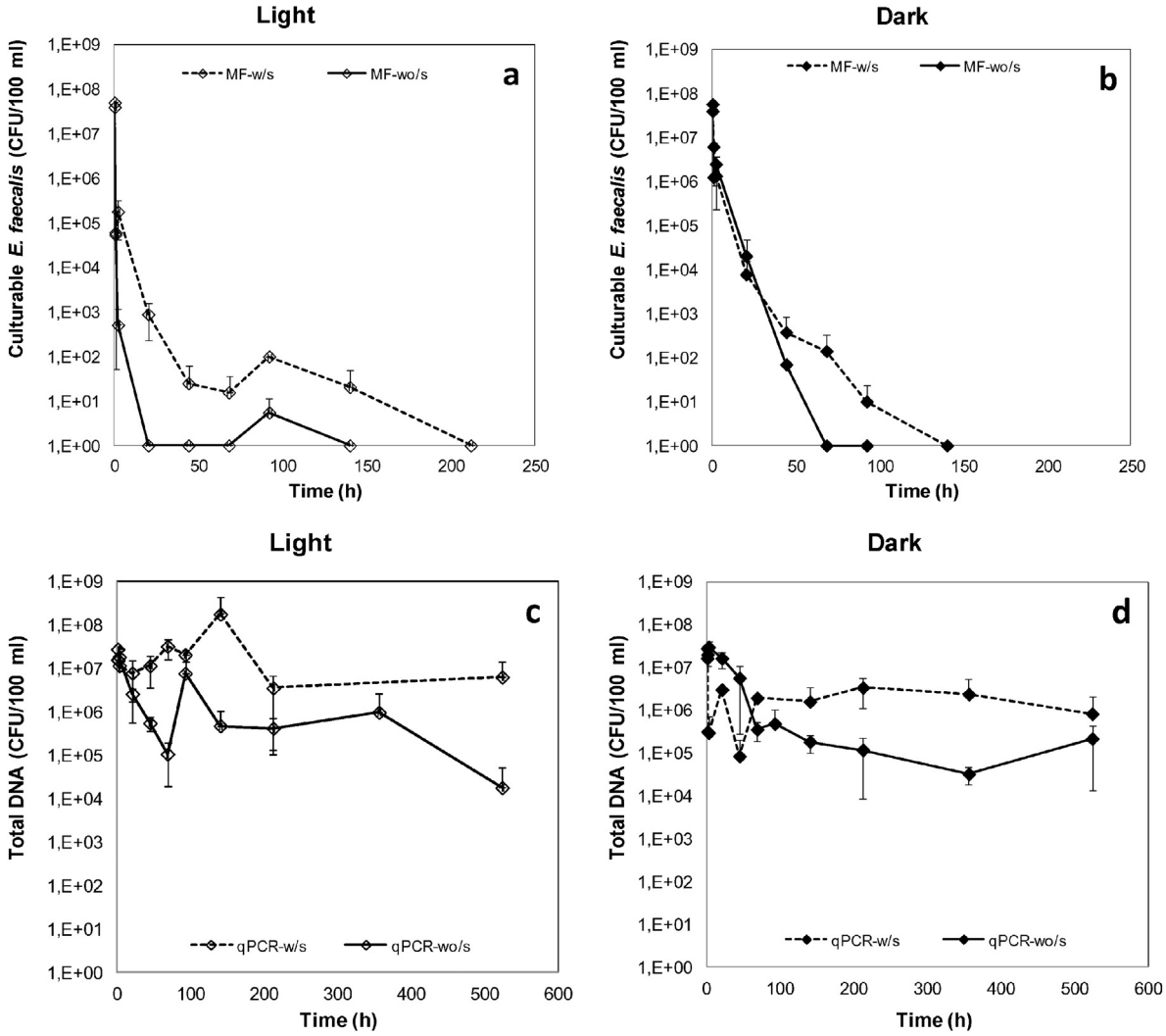

Fig. 3. Decay curves for culturable *E. faecalis* (a, b) and its total DNA (c, d) exposed to sunlight (a, c) and in the dark (b, d), with (w/s) and without (wo/s) solid particles. Enumeration was done by two methods: membrane filter technique (MF) and qPCR.

Inactivation of culturable *E. faecalis* without solid particles in the light occurred within the first 20 h (Fig. 3a). Instead, the cells in the presence of solids received some protection from them, persisting for longer periods (Fig. 3a). In the dark, this indicator in the absence of solid particles disappeared after 68 h (almost 3 days) (Fig. 3b), and with particles it seemed to survive longer (Table 3).

In the dark with solid particles (best situation for the survival of microorganisms) *E. faecalis* was almost 5 times more persistent than in the light without solid particles (worst situation for microorganisms) (Table 3), reaffirming the protective role that solid particles exert on microorganisms survival. In the dark, inactivation may be attributed to other environmental stressors present in water. These kinetic constants rates values found in the container in the dark were similar as the ones reported by Bae and Wuertz (2009) in light and dark (0.2363 and 0.119 h^{-1}, respectively) but in that case microcosm bags were prepared using seawater and without solids particles.

Bacteria become unculturable (although they may remain active) when exposed to environmental stressors such as starvation, temperatures out of the range of growth, elevated osmotic pressure, low oxygen concentration, and exposure to sunlight; however, they may remain metabolically active. These factors cause the decrease in colony counts of culturable cells (Oliver, 2005).

Even if DNA extraction was done to avoid inhibition during qPCR amplification, the concentration of solid particles present in the samples was so high that the problem persisted due to the co-extraction of inhibitors, probably humic acids. Therefore, in many of the undiluted samples tested, no amplification signal was obtained (false negative) although it was known that the concentration of target DNA was greater than the detection limit established for *E. faecalis*. To remediate this, the dilution approach proposed by Rajal et al. (2007) was used. Serial dilutions of each sample that presented no signal were done, up to the point where inhibitors did not affect DNA amplification anymore (Rajal et al.,

Table 3
Kinetic parameters for the inactivation of culturable *E. faecalis* and the decay of its total DNA exposed to sunlight and in the dark, with (w/s) and without (wo/s) solid particles. Enumeration was done by two methods: qPCR and membrane filter technique (MF).

Condition	Method	Solids	k_{si} (h^{-1})	$t_{1/2}$ (h)	T_{99} (h)	R^2
Sunlight	MF	wo/s	0.6392	1.1	7.2	0.6891
		w/s	0.2526	2.7	18.2	0.7670
	qPCR	wo/s	0.042	16.5	109.6	0.9161
		w/s	0.0379	18.3	121.5	0.8710
Dark	MF	wo/s	0.2363	2.9	19.5	0.9669
		w/s	0.119	5.8	38.7	0.8618
	qPCR	wo/s	0.029	23.9	158.8	0.8379
		w/s	0.0041	169.1	1123.2	0.7268

k_{si}: inactivation kinetic constant, $t_{1/2}$: half-reduction time (time for 50% reduction), T_{99}: time for 2-log reduction, R^2: coefficient of determination.

2007). The dilution factor was later taken into account to back calculate the total DNA amount.

Since nucleic acids can remain in the environment and still be detected by qPCR after the microorganism has lost its viability, it is important to know their persistence in water as separate entities from the intact cells (Walters et al., 2009). In this sense, decay curves obtained from qPCR and the traditional culture-based method (MF) were quite different in both light and in the dark (Fig. 3), despite the nucleases that may be present in the water. In addition, T_{99} were much higher and decay constants rates much lower than for culturable cells, both in light and dark (Table 3). Total DNA of *E. faecalis* persisted for longer periods of time than culturable cells, remaining almost constant until the end of the experiment (Fig. 3c, d; Table 3). DNA persistence exposed to sunlight compared to culturable cells was also observed by other authors (Bae and Wuertz, 2009; Walters et al., 2009).

As it can be seen here, qPCR does not necessarily produce results comparable to a culture-based method. Because qPCR has been proposed by the new legislation to monitor enterococci in waters (EPA, 2010), rather than or along with culture-based methods, it is necessary to understand how often and under what circumstances these two methods will bring different results (Boehm and Sassoubre, 2014; Converse et al., 2012).

Although DNA can persist longer than culturable cells when exposed to light, this persistence was higher in the dark both with and without solid particles (Fig. 3c, d). The time required for DNA disappearance in the light without particles was 1.5 lower than in the dark and 9 times lower when solid particles were present (Table 3). Moreover, in the absence of solid particles, DNA exposed to both sunlight and in the dark decay faster than in their presence (Table 3). This could mean that solid particles also exert a protective role on free DNA or DNA inside damaged cells or in active but non-culturable cells (Walters et al., 2009), even if this susceptibility occurs in longer periods of time. The number of *E. faecalis* obtained by qPCR in both light and dark condition in the absence of solid particles decreased two-log units during the first 100 h but then they remained constant until the end of the experiment (Fig. 3c, d). This behavior was also observed by other authors (Bae and Wuertz, 2009).

Due to the persistence of DNA in the range of several days to weeks (or even longer periods of time), after cell death, the methods based on the quantitative determination of DNA as qPCR can lead to an overestimation of the microorganisms and harmful pathogens (Nocker and Camper, 2006). To address this problem, technical feasibility based on the use of dyes for differentiation of viable and non-viable by fluorescence microscopy or dyes such as propidium monoazide (PMA) in combination with qPCR (Bae and Wuertz, 2009) can be used. However, these techniques need to be optimized yet.

Still, the presence of a large amount of DNA in water with high persistence is of concern and carries out a risk, especially in the case of pathogens or other organisms carrying antibiotic resistant genes. Free DNA could be incorporated into other bacteria in the aquatic environment by transformation, giving particular characteristics and genetic changes that would impact on people and animal health (Hong et al., 2013). DNA that contains mobile antibiotic resistant genes could be released into the environment from lysed cells. This extracellular DNA may be adsorbed onto solid particles and natural organic matter and, therefore, can persist longer in the environment and easier be transported and eventually incorporated by other bacteria (Hong et al., 2013). Although antibiotic resistant genes associated with extracellular DNA can also be inactivated by sunlight, little is known yet about this phenomenon.

Finally, it is important to say that even though in this study some variables were not analyzed, the results obtained are quite important to the field. First, the experience was carried out outside the laboratory (natural sunlight and dark, day and night temperatures), with intact environmental water and solids; so microorganisms under study were exposed to conditions close to reality. This permitted us to obtain more reliable kinetic constant rates and inactivation times. On the other hand, it was possible to calculate not only decay constant rates for free DNA but also for DNA attached to solids. The latest has not been often done because it involves a great challenge since the presence of inhibitors in environmental samples can interfere with qPCR. However, most microorganisms and DNA in aquatic environments are interacting with solid particles and other components present in water, which give them protection. Therefore, this situation cannot be ignored, and more research related to this field must continue in order to obtain robust values that will help achieving better results in risk assessments simulation models.

4. Conclusions

Both *E. coli* and *E. faecalis* are inactivated more rapidly when exposed to sunlight than in the dark, as shown in their higher kinetic constant rates and lower reduction times. In turn, these bacterial indicators survived longer in the presence of solid particles than without them, demonstrating their protective effect. However, inactivation of *E. faecalis* was more detrimental than *E. coli* in all cases. Water composition, type, location, and uses, climate change, sunlight intensity, indigenous microbiota, and predators could be the reason for the difference in their survival. All these details must be put under consideration when analyzing their behavior and the risk involved.

E. faecalis detection by qPCR showed that the persistence of DNA was higher than that of culturable cells. In this case sunlight also accelerated DNA decay and solid particles exerted a protective role. This indicates that persistent DNA might move to other parts of the environment and could subsequently be detected as an unknown pollution source, becoming a potential health risk. It is important to keep in mind that qPCR allows the detection of total DNA, but do not account for viable cells, even after the cell has lost its viability due to its high persistence in the environment. In addition, inhibition problems and detection limits are still issues that need further research to obtain in the future, more reliable results.

The understanding of these results regarding the application of different methodologies, as well as the effect of different kinetic parameters depending on the conditions, can be used in QMRA and also in the improvement of water treatment processes, determining the inactivation efficiency by sunlight. On the other side, these values may be included in future water quality models, to assess the fate and transport of microorganisms in different aquatic environments.

Summarizing, the results obtained here allow us to tell that if indicators are measured using culture-based methods then these results will reflect recent contamination since sunlight inactivation is relatively fast. However, the measured numbers will be an underestimation of the real contamination since damaged or non-culturable cells are not detected. On the other hand, if the detection is performed by qPCR the results will represent an overestimation of the contamination due to the high persistence of DNA in the environment. These situations must be taken into account during the decision making process.

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