



# *Escherichia coli* Capacity to Repopulate Microcosms Under Osmotic/ U.V. Synergic Stress in Tropical Waters

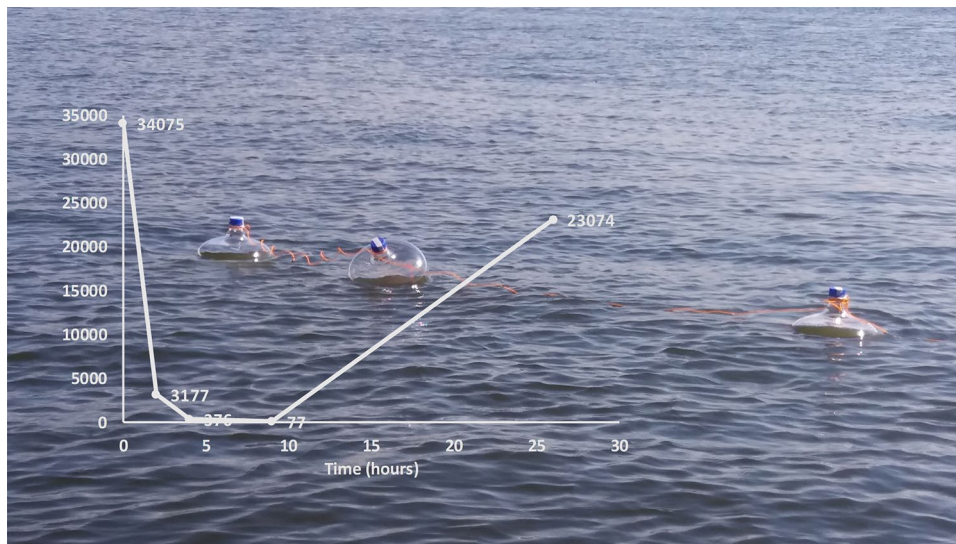
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## Abstract

In both Brazilian and European regulations, the impact assessment of sewage discharges into coastal waters is based on microbiological analyses of fecal indicators such as *Escherichia coli*, frequently used in prevision hydrodynamic models. However, the decay rates of *E. coli* vary depending on environmental conditions, and analysis may lead to inaccurate conclusions. This study aimed to analyze the decay of culturable and viable (but not culturable) *E. coli* in outdoor conditions, by creating microcosms inoculated with pre-treated sewage. The microcosms were filled with 9.88 L of filtered water (0.22 µm membrane), 3.5% salt, 0.1–0.2% BHI, and 1% bacterial suspension obtained by reverse filtration. PMA-qPCR of *E. coli* uidA gene and Colilert measurements were applied to evaluate population counts after 2 h, 4 h, and 26 h. After nine hours of exposure to solar radiation, the viable cells decreased to 2.76% (interpolated value) of the initial population, and the cultivable fraction of the viable population accounted for 0.50%. In the dark period, the bacteria grew again, and viable cells reached 8.54%, while cultivable cells grew to 48.14% of initial population. This behavior is possibly due to the use of nutrients recycled from dead cells. Likewise, populations of *E. coli* in sewage outfalls remain viable in the sediments, where resuspension can renew blooming.

## Graphic Abstract



**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00284-020-02319-2>.

Extended author information available on the last page of the article

## Introduction

The contamination of coastal areas by domestic effluents poses a health threat associated with bathing [1, 2]. Furthermore, the emergence of antibiotic resistance in pathogenic bacteria—which also reach the environment through the disposal of sewage—has increased the risk of infectious diseases [3, 4]. For example, 11.2% of the total *Escherichia coli* isolates from aquatic ecosystems surrounding the megalopolis of Rio de Janeiro hosted molecular markers of diarrhea genes, and 37% were resistant to at least one antibiotic among the 11 tested [5]. Thus, with increasing sewage production in large urban areas, sewage outfall positioning has become crucial to minimizing human contact with plumes. In these areas, further studies on bacterial decay may yield more reliable hydrodynamic models, allowing the ideal positioning of sewage outfalls, reducing the health hazards associated with fecal contamination of the environment [6].

Decay studies are usually conducted in microcosms or mesocosms, and consist of experiments where bacteria cultures [7, 8] or human and animal feces [9] are inoculated with variable concentrations of bacteria and large amounts of organic matter as much as 5% and up to 50% [10, 11]. Special care must be taken, though, to determine not only indicator organisms, but also the origin and physiological status of the inoculated strains. The use of culture-based inoculants in microcosms requires that the experiment starts in the stationary growth phase, when nutrients are scarce [12]. On the other hand, feces, or *in natura* sewage inoculation introduces concentrated nutrients, inducing bacteria to grow again in a log phase [13]. Furthermore, other contaminants may be present in the sewage that can impair molecular analysis [14], an issue that was resolved by Carneiro et al. [15] who developed the reverse filtration (RF) technique and obtained a suspension of uncultivated *E. coli*, supplemented with only 0.1% BHI. This inoculum proved to be physiologically appropriate for decay microcosms since it did not lead the microcosms to bloom, neither to a total population crash, allowing to control the interfering stressors. Besides, it has produced a clarified inoculum that was shown to be appropriated for molecular analysis.

Bacterial decay rates are frequently expressed as  $T_{90}$ , which represents the time required to reduce the bacterial population by 90% of its original amount. The quantitative evaluation of  $T_{90}$  on a spatial basis is the best information for determining the disposal location and defining the boundaries for safe bathing zones [16–18]. Although these studies are not recent, it is becoming consensus that in cases where evaluations are based on culture analyses (e.g., Colilert®), the results reflect the decline in

culturability instead of viability of bacteria life, a limitation which has been a common obstacle to significance in these studies [19–21]. As culturability declines faster than viability, these approaches result in underestimations of  $T_{90}$ , making the scope of the modeling inadequate.

On the other hand, the decay of molecular markers measured by real-time quantitative PCR (qPCR) analyses makes no distinction between viable and dead cells in longer decays. Furthermore, molecular markers are little affected by solar radiation [10, 11, 22]. The molecular methodology of analysis with the nucleic acid intercalating Propidium Monoazide (PMA) dye, coupled with qPCR (PMA-qPCR), however, is capable of distinguishing between living and dead bacterial cells [23]. This technique can reduce the false-positive results from non-viable bacterial cells and can count those that are viable while not culturable. Moreover, according to Takahashi et al. [24], the use of optimal concentrations of SDS (Sodium Dodecyl Sulfate) surfactant in PMA-qPCR (known as SDS-PMA-qPCR) reduces false positives due to non-viable bacteria with membranes that are impermeable to PMA.

Some researchers have used PMA to evaluate other species decay, as Bacteroidales [25–28], or, though the PMA have been used for other groups, the *E. coli* were measured by enzymatic method [9, 26, 28, 29]. Either, some of them applied PMA to evaluate bacterial decay in sediments or freshwater [30, 31]. There are also some studies that did not established any microcosm arrangements, just *E. coli* counts in raw sewage, with no comparisons with initial counts [32]. Thus, although this technique is already well known, works using PMA-qPCR to measure *E. coli* decay in microcosms under natural conditions are still scarce.

Although PMA-qPCR has constituted a relevant advancement for quantitative analyses of bacteria, choosing the appropriate molecular marker is critical for increasing the sensitivity and specificity of the procedure. The gene *uidA* has been chosen as a target for the molecular detection of *E. coli* in water quality surveys and monitoring [14, 33–36]. Among the 27 genes belonging to the pan-genome of *E. coli*, *uidA* is one of the three most frequently observed in the environmental clade [37].

The purpose of this research was to assay the decay of viable cells of *E. coli*, in order to better understand the effects of the synergic stress of sunlight and salinity in the survival. The assays were carried out in microcosms, simulating tropical seawater, inoculated with sewage, processed by the RF technique, and quantified by PMA-qPCR of the *uidA* gene.

## Materials and Methods

The microcosm experiments were assembled under natural environmental conditions (in-situ). The raw sewage samples for all assays were collected at the input of a sewage

treatment plant in Rio de Janeiro, Brazil, after they had passed through the sand grit chamber but before any treatment had been applied.

### Microcosms in Environmental Conditions of Guanabara Bay, Brazil

Eight microcosms were prepared: set 1 (A, B, C, and D), and set 2 (E, F, G, and H), containing a solution of 9.88 L of 0.22- $\mu\text{m}$  membrane-filtered (Millipore brand) tap water. The microcosms were supplemented with 0.1% BHI and sterile NaCl (P.A.), reaching a final concentration of 3.5% (similar to seawater). Microcosm “H” was supplemented with 0.2% BHI with the aim of evaluating whether nutritional supplementation was sufficient. Since during environmental expositions, the incidence of natural UV light in closed vials may promote significant increases in temperature in a short period, thereby modifying the conditions, we decided to carry out these experiments in large (10 L) transparent polycarbonate Thermo brand (Waltham, MA, USA) bottles, increasing their thermal capacities.

For each of the natural environmental experiments (sets 1 and 2), 440 mL of raw sewage was processed by RF [15], yielding a colorless suspension inoculum containing *E. coli*. The suspension was supplemented with 0.44 mL BHI and maintained at 20 °C until 08:00 AM of the subsequent day. The suspension was then homogenized and divided in four 110-mL aliquots. Ten mL of each aliquot ( $t_0$ ) was taken and sent to the Laboratory of Comparative and Environmental Virology of Institute Oswaldo Cruz (IOC—Fiocruz) for the initial analyses of PMA-qPCR. The remaining 100-mL aliquots were used to inoculate, in a proportion of 1%, each microcosm, carried out in Gragoatá beach in Guanabara Bay, Rio de Janeiro, Brazil, in a sunny day.

After inoculation and homogenization of the large bottles, 200-mL samples were taken at the beginning ( $T_0$ ) of sunlight exposure, after 2 h ( $T_2$ ), 4 h ( $T_4$ ) for colimetry and chemistry, and 26 h ( $T_{26}$ ) for final analyzes of the colimetry, chemistry, and PMA-qPCR. The samples were properly conditioned and transported to the laboratory in refrigerated containers. The samples were reverse filtered (as described below), for analysis by PMA-qPCR, which enables the distinction of genetic material in living and dead cells. Although the microcosm was maintained under solar radiation up to 5:00 p.m. ( $T_9$ ), we were unable to run PMA-qPCR from samples from that last time.

The reverse filtration (RF) as described by Carneiro et al. [15] consists of sampling raw sewage samples from upstream from a sewage treatment plant and passing it through a 0.80- $\mu\text{m}$  pore membrane—Millipore brand. The filter is discarded, and the filtrate is passed through a 0.45- $\mu\text{m}$  membrane. The membrane of the second filtration (0.45  $\mu\text{m}$ ) is placed with the top face down in the filtration

set. The filter is leached with the same volume as the initially filtered sewage, but with sterile phosphate water, resulting in a suspension of bacteria with reduced concentrations of contaminants, dissolved solids, and nutrients.

Solar radiation data were collected with a Delta OHM radiometer (Padua, Italy), model HD-2302 with a LP-471-UVA probe, in the spectral range between 315 and 400 nm, with a peak at 360 nm, in periods of 30–40 min. An Onset Hobo Pendant temperature data logger (San Jose, USA) was placed inside bottles A and E, recording internal water temperature every 15 min. Both Total Organic Carbon analyses (performed with an Ion-Chromatographer) and quantitative colimetric analyses [performed on the same day with the defined substrate method—Colilert® (COTIA, Brazil)] of all samples were done in the Laboratory of the Department of Environmental Sanitation and Health (Fiocruz).

### Molecular Analysis

For the detection and quantification of *uidA* genes from *E. coli*, the samples were prepared according to Takahashi et al. [17] with modifications, in three steps:

#### (1) Samples Concentration and Treatment with SDS-PMA

From the initial filtrate obtained by RF ( $T_0$ ), 10-mL samples were centrifuged at 6500 $\times g$  for 22 min at 15 °C. From the final filtrate ( $T_{26}$ ), 50-mL aliquots of RF were centrifuged at 3150 $\times g$  for 40 min at 15 °C. The supernatant was carefully discarded, leaving a volume of 450  $\mu\text{L}$  solution/pellet. The pellets were transferred to 1.5-mL tubes, and a 25- $\mu\text{L}$  sodium dodecyl sulfate (SDS) solution was added, creating a final concentration of 200 ppm. The tubes were vortexed and incubated at 37 °C for 15 min. In a dark room, the samples were pre-treated with PMA (Biotium Inc., Hayward, CA) in a concentration of 10  $\mu\text{M}$ . The microtubes were kept in the dark for 6 min and homogenized in a shaker at 200 RPM, followed by photolysis of dye with a PMA-LiteLED Photolysis Device—Biotium®, (Fremont, USA)—for 10 min. Next, the samples were centrifuged at 15,000 $\times g$  for 3 min. The supernatant was discarded, and the pellets were re-suspended in 50  $\mu\text{L}$  of ultrapure water and stored in a freezer at – 70 °C for later genetic analysis.

#### (2) DNA Extraction

The stored genomic material obtained from the pellets was extracted with a commercial Genomic DNA kit NucleoSpin from Tissue Macherey–Nagel, (Düren, Germany), following the manufacturer’s protocol for bacteria.

### (3) SDS-PMA-qPCR

The gene *uidA* (GenBank access number: S69414.1) was the genetic target for the detection and quantification of *E. coli* in the samples of the microcosms. The reaction (total of 25 µL) was carried out with 5 µL of extracted genomic DNA and 1 × TaqMan Universal Master Mix II, with UNG (Applied Biosystems™, Foster City, USA). In addition, the primers EC-784F and EC-866R (Invitrogen, Brazil) and the probe EC-807 (Integrated DNA Technologies,™ Brazil) sequences for the detection of *E. coli* were used in the final concentrations of 10 µM and 5 µM, respectively, according to Frahm, Obst [33]. The probe was labeled 6-F AM-5' with quencher TAMRA-3'. The qPCR was performed in the ABI PRISM 7500 Real-Time TaqMan System (Applied Biosystems™ Foster City, USA). The thermal cycling conditions included an initial UNG activation for 2 min at 50 °C, followed by denaturation for 10 min at 95 °C and 40 cycles of denaturation at 95 °C for 15 s each and 1 min at 60 °C for hybridization. The standard curves were designed with gBlock Gene Fragments (Integrated DNA Technologies™ Brazil). The whole experiment was performed in duplicate, including negative and positive controls of the nucleic acid as well as No Template Control (NTC). No serial dilution samples were used because the samples obtained from RF removed most of interfering contaminants. Samples that showed signals crossing the threshold line in both replicates until  $Ct \leq 38$  presenting a characteristic sigmoidal curve was considered positives. The results of qPCR standards and samples were plotted in graphs and are presented in supplementary materials SM 1 and SM 2, respectively.

### Data Analysis

According to the previous work of Feitosa et al. [16], neglecting the diffusive and advective terms of the mass conservation equation, the bacterial die-off varies over time ( $t$ ) following a first-order decay according to Eq. (1). According to these authors, the  $k$  decay rates can also be expressed by the parameter  $T_{90}$  as shown in Eq. 2.

$$C = C_0 e^{-kt} \tag{1}$$

$$T_{90} = \frac{2.3}{k} \tag{2}$$

From both equations, the " $k$ " is the decay rate of populations of *E. coli* from the second group of microcosms (E, F, G, and H), " $t$ " is the exposure time of the cells. " $C$ " is the concentration of cells at a given time " $t$ ." " $C_0$ " is the initial concentration of *E. coli*, and " $e$ " is the neperian number.

The  $k$  values were based on samplings and were obtained initially for times  $T_0, T_2, T_4,$  and  $T_{26}$  hours for culturable cells, and  $T_0$  and  $T_{26}$  for viable cells. Considering that the samples from set 1 did not form pellets, the average qPCR values of the set 2 samples were applied to Eqs. 1 and 2 to reach the viable  $k$  and  $T_{90}$  values. The assay "H" for the initial sample ( $T_0$ ) also did not result in pellets, and it was filled up from the average  $k$  value from assays E, F, and G. Therefore, the geometric mean values of the populations for each time ( $T_n$ ) and the arithmetic mean value of each  $T_{90}$  were considered for generalizations.

We interpolated counts for  $T_9$ , based on a study with similar inoculum/microcosm in dark conditions, which estimated a population growth of 300 times between the 9th h up until the 26th h [15]. Hence, in the present study, the cultivated population at  $T_9$  (final of sunlight exposure period = 9th h) was estimated as equal to that observed at  $T_{26}$  divided by 300 ( $C_{T_9} = C_{T_{26}}/300$ ), overlooking eventual inactivation, occurred after exposure to the sun.

## Results and Discussion

The temperatures in the microcosms and the sea differed by up to 7.51 °C in the first set (A–D), with an average difference of 3.0 °C, and up to 2.52 °C in the second set (E–H), with an average difference of 0.24 °C. The bottle walls attenuated the natural solar radiation, functioning as an artificial turbidity, in comparative way as the water column do when it is above the sewage plume [6]. When the radiation was perpendicular to the side of the containers, the attenuation was 36.26% ( $n = 17, R^2 = 0.894$ ), and when the incidence was perpendicular to the bottom of the container, the attenuation was 49.18% ( $n = 17, R^2 = 0.995$ ). The data of the assays made under sunlight are shown in Table 1, and the temperature and radiance are presented in SM 3a and SM 3b.

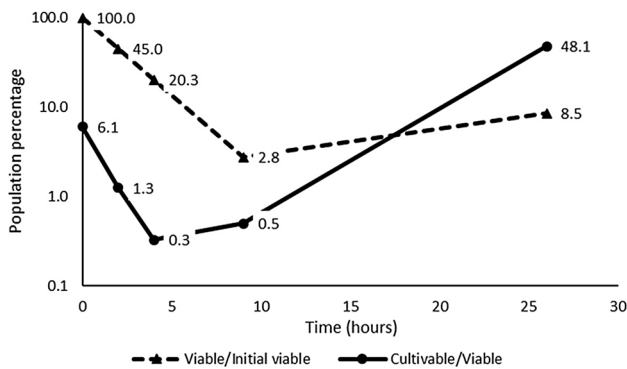
For both the sets exposed to sunlight, the RF method was able to obtain a wild bacteria inoculum and pollutants reduction for the molecular analysis. It was observed, however, that in the lower concentrations of  $10^4$  culturable cells, verified in the first set (A, B, C, and D assays), there was no pellet formation. This result possibly indicates that impurities and cell mass would be too low to help in the formation of pellets. Even so, its results are shown here because they confirm the standard of decay and regrowth of the culturable population observed in the second set of microcosms. The second set of assays (E, F, and G)—with the higher initial concentration of cells ( $10^4$ )—did show the formation of pellets. Thus, the RF could be more effective for microcosms decays assays with controlled initial concentrations than to monitoring ones, when the sensibility to smaller concentrations is required [14].



**Table 1** Microcosm *E. coli* populations measured by the Colilert® culture method and by PMA-qPCR throughout the experimentation

Assay	Colilert® culture by incubation period (MPN cells 100 mL <sup>-1</sup> )				PMA-qPCR (cells 100 mL <sup>-1</sup> )	
	<i>T</i> <sub>0</sub> initial	<i>T</i> <sub>2</sub> (2 h)	<i>T</i> <sub>3</sub> (4 h)	<i>T</i> <sub>4</sub> (26 h)	<i>T</i> <sub>0</sub> initial	<i>T</i> <sub>4</sub> final (26 h)
A	2.75 × 10 <sup>3</sup>	<1	<1	4.0 × 10 <sup>1</sup>	No pellets	No pellets
B	2.90 × 10 <sup>3</sup>	<1	<1	1.7 × 10 <sup>3</sup>	No pellets	No pellets
C	3.90 × 10 <sup>3</sup>	<1	<1	2.5 × 10 <sup>2</sup>	No pellets	No pellets
D	2.95 × 10 <sup>3</sup>	<1	<1	1.3 × 10 <sup>3</sup>	No pellets	No pellets
E	3.60 × 10 <sup>4</sup>	1.90 × 10 <sup>2</sup>	1.70 × 10 <sup>2</sup>	>2.42 × 10 <sup>4</sup>	6.00 × 10 <sup>5</sup>	6.70 × 10 <sup>4</sup>
F	3.30 × 10 <sup>4</sup>	4.10 × 10 <sup>3</sup>	2.00 × 10 <sup>3</sup>	2.00 × 10 <sup>4</sup>	1.15 × 10 <sup>6</sup>	2.43 × 10 <sup>4</sup>
G	4.00 × 10 <sup>4</sup>	3.20 × 10 <sup>4</sup>	5.80 × 10 <sup>1</sup>	>2.42 × 10 <sup>4</sup>	4.70 × 10 <sup>5</sup>	1.38 × 10 <sup>5</sup>
H	2.80 × 10 <sup>4</sup>	4.00 × 10 <sup>3</sup>	1.00 × 10 <sup>3</sup>	>2.42 × 10 <sup>4</sup>	3.06 × 10 <sup>5</sup>	3.41 × 10 <sup>4</sup>

MPN most probable number (calculated according to Colilert® manufacturer's instructions and table)



**Fig. 1** Decay values of viable cells in percentages from the initial value and percentages of cultivable cells from viable cells during microcosms' experiments (mean values from experiments E, F, G, and H). Viable cells percentages were obtained by PMA-qPCR, while cultivable percentages were obtained by the culture method. Samples were collected in times 0 h, 2 h, 4 h, and 26 h. Values for the 9th h were interpolated. ANOVA between different experimental microcosms showed insignificant differences ( $P < 0.07$ )

The low proportion of cultivable/viable cells (6.07%) since the beginning of sun exposure (Fig. 1) shows that the population was under stress, possibly due to exhaustion of the 0.1% nutrients added the previous day, in agreement with Carrillo [38].

**Table 2** Microcosm decay rate ( $-k$ ) values from set 2, measured by the culture method and by PMA-qPCR throughout experimentation

Assays	Culturability					Viability	
	0–2 h	2–4 h	0–4 h	0–9 ha	9–26h <sup>b</sup>	0–9 ha	0–26 h
E	-2.612	-0.057	-1.334	-0.678	0.336	-0.310	-0.088
F	-1.046	-0.359	-0.702	-0.690	Growth	-0.696	-0.152
G	-0.112	-3.157	-1.634	-0.690		-0.170	-0.051
H	-0.977	-0.693	-0.835	-0.651		-0.420	-0.088
<i>k</i> <sub>average</sub>	-1.186	-1.066	-1.126	-0.677		-0.399	-0.095
<i>T</i> <sub>90</sub> average	1.94	2.16	2.04	3.40		7.43	28.19

<sup>a</sup>Estimated values under solar radiation

<sup>b</sup>Estimated values in the dark

The decay rates ( $k$ ) in each microcosm are shown in Table 2, where the assay G showed a different population evolution from the others, strongly decaying after the 2nd h after inoculation.

In the first microcosm set, due to the low initial concentration of cells, it was not possible to calculate the specific decay rates, because in the first two hours, the culturable fraction was already below one cell 100 mL<sup>-1</sup>. However, in both cases, the  $T_{90}$  of the culturability was smaller than 2 h, with the average  $T_{90}$  of the second set equaling 1.94 h. These results of culturable *E. coli* decay under solar radiation in 3.5% of NaCl-treated microcosms were consistent with results described in the literature, under similar conditions [16, 17]. The results obtained after the end of the synergistic period (salinity and solar radiation— $T_9$ ), indicate a significant increase in population in the dark, until the last sampling (26th h), reaching populations of up to 10<sup>3</sup> cells 100 mL<sup>-1</sup> in the first set, and greater than 2 × 10<sup>4</sup> cells 100 mL<sup>-1</sup> in the second set. This process is explained by not only the recovery of culturable *E. coli*, but also resuscitation of VBNC, which was discussed by Pinto et al. [39] as the result of the improvement in their physiological condition. In resuscitation, cell division is interrupted during incidence of solar radiation, but regrow in the dark phase from the stress-resistant fraction (Fig. 1) [39, 40]. In the present

research, we could not distinguish what is the contributions of culturable and VBNC to growth of the dark phase, but it is reasonable to think that both states are important. Under extreme radiation incidence, sterilization of the water (complete elimination of culturable cells) should not avoid growth in the dark phase.

Bacterial decays in the environment usually show an inflection indicating biphasic die-off, as observed by Zhang et al. [41], and, from this work, shown in Fig. 2, after 2 h and 4 h of exposure. In the first 2 h, the average decay rate was strongly influenced by  $k$  from assay E ( $-2.612 \text{ h}^{-1}$ ), and less from assay G ( $-0.112 \text{ h}^{-1}$ ), but in the second inflection, these contributions were reversed ( $-0.057 \text{ h}^{-1}$  and  $-3.157 \text{ h}^{-1}$ , respectively). Microcosm G was the only one where dissolved organic carbon (DOC) concentrations decreased near the end of the experiment (SM 4), probably because growth of viable *E. coli* attaining  $10^5$  cells. Overall, there was a strong correlation ( $r=0.96$ ;  $p<0.05$ ) between DOC consumption and  $k$  values. Once the microcosms evaluated in this study were similar (0.1% BHI, except for the assay H, with 0.2% BHI) under the same environmental conditions, the decay differences could be associated with genotypically different subpopulations, as well as the proportions between them in each bottle. This agrees with Bucci et al. [40] who stated that some adapted subpopulations occupy unpopulated surface waters leading to biphasic growth pattern.

The viable cells presented an average  $T_{90}$  of 7.43 h under solar radiation and average  $k$  rate of  $-0.399 \text{ h}^{-1}$  or  $-0.095 \text{ h}^{-1}$  computed over the entire experimental time (Table 2). In Fig. 2, it is observed that there was an estimated percentage of 2.76% of the population surviving after 9 h of sunlight and saline synergism. Nevertheless, under dark conditions, the total population increased, reaching 8.54% of

the initially inoculated, while the culturable cells decreased from 6.07 to 0.33% in the 4th hour but increased to  $>48.1\%$  of the final viable population.

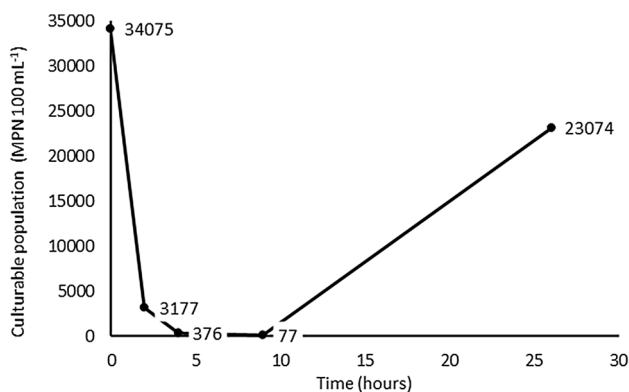
The in-situ microcosms reproduced different conditions of outfalls regarding plume depths and dispersion rates of bacteria and nutrients. These experiments always start on surface water, enhancing the action of solar radiation [16]. On the other hand, since the inoculum had just 0.1–0.2% of BHI broth, the rapid decay rates would favor the recycling of the organic matter (produced by the bacteria itself), fertilizing the system, and increasing bacterial growth in the dark. Similar results are described in the literature [40, 42]. The estimated growth of viable cells in the dark between 9 and 26 h represents a cryptic growth, favored by this recycling. In this sense, before the microcosm was exposed to sunlight, the number of viable cells was almost 16 times that of the culturable ones, reaching 302 times in the 4th h, and dropping to  $<2.1$  times by the end of the 26th h. This process was possibly carried out by Gaspers cells, as stated by Bucci et al. [40]. It seems that in assay G, growth was stronger, because greater amounts of carbon were assimilated (SM 4).

Even though comparisons of the results of culturable cells and sunlight showed the importance of the radiation, no statistical correlation was found. The lack of correlation can be explained by the fact that prevalent radiation affected genetically and physiologically different subpopulations in each microcosm. Furthermore, although the synergism between radiation and salinity is important for decay, here the diversity of natural *E. coli* would have been fundamental to maintain a viable cell stock in the environment, as discussed by van Elsas et al. [43].

## Conclusion

The application of reverse filtration was shown to reliably evaluate solar radiance and osmotic stress effects on decay of viable *E. coli* cells into microcosms. The procedure yielded a clean sample that favored the molecular target amplification with the PMA-qPCR of *uidA* gene, plus SDS and reducing both false-positives and false-negatives results. This protocol can be applied to a number of situations, to provide a better understanding of the environmental fate of viable *E. coli* in marine waters.

The analysis of the decay of viable *E. coli* in outdoor conditions, using microcosms inoculated with RF sewage, showed that after nine hours of exposure to solar radiation, viable and culturable cells strongly decay, but in the dark period, both bacterial states grew again and repopulated the microcosm. Although the microcosms did not allow natural exchanges of dissolved material with the surrounding, as in a real environment, it was possible to infer the cumulative implications of continuous inflow of nutrient-rich sewage



**Fig. 2** Mean culturable population decays obtained from microcosms E, F, G, and H. Values were obtained by the culture method of samples collected in times 0 h, 2 h, 4 h, and 26 h. Values for the 9th h was interpolated. ANOVA between different experimental microcosms showed insignificant differences ( $P<0.07$ )

plumes in closed, or sheltered, environments such as lagoons and bays. Besides, the study allowed identifying the limited capability of solar radiation for disinfection on bacterial populations.

Although the culturable cells'  $T_{90}$  values were within the range of two hours, viable cells'  $T_{90}$  values were 7.26 h. Furthermore, the remaining fraction of viable cells not only survived, but also grew, with an increase in culturable/viable cells proportion. Culturable and viable *E. coli* decays were inversely correlated with DOC consumption. Thus, the availability of nutrients and the assimilation capacities of the bacteria in the microcosms proved to be equally or more important than the environmental stressors. This phenomenon could occur when levels of organic matter remain for long periods, both in point (like outfalls) and diffuse sources. From this view point, it can be stated that the  $T_{90}$  of viable cells should be taken into consideration in defining location of outfalls, instead of culturable cells alone. Furthermore, colimetry (as measured by Colilert) cannot be treated as a simple semi-conservative variable in hydrodynamic models.

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**Authors Contributions** MTC: Responsible for conducting the research; author of the first draft; participated in the laboratory and field work. DVP: Advisor of the research; conception of the research; contributions in the manuscript; helped in the laboratory work. RCF: Advisor of the research; contributions in the manuscript; helped in the laboratory work. LGPM: contributions in the manuscript; helped in the laboratory work. MPM: Advisor of the research; contributions in the manuscript; helped in the laboratory work. JCW: Advisor of the research; conception of the research; contributions in the manuscript; participated in the laboratory and field work.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they adhere to the ethical responsibilities established in the IFA of Current Microbiology and that they do not present any conflict of interest regarding this article.

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