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Differential decay and prediction of persistence of *Enterococcus* spp. and *Escherichia coli* culturable cells and molecular markers in freshwater and seawater environments

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ABSTRACT

To quantify the impact of fecal pollution on the microbiological bathing water quality, predictive modeling is being increasingly used in which the decay rate of the fecal indicators plays an important role. The decay of sewage-sourced enterococci and *Escherichia coli* culturable cells and their associated molecular markers (16SrRNA) quantified by Quantitative Reverse transcription PCR were measured in controlled microcosms as well in *in situ* conditions using different water types, from marine waters to fresh waters with intermediate salinity. All bacterial decays were fitted to a first order decay model. In the laboratory study, the light radiation was the most influent factor affecting *E. coli* and enterococci survival by culture methods although environmental conditions weakly impacted the decay of molecular markers. The results also indicated differential persistence of genetic markers and culturable organisms of fecal indicator bacteria in different water systems. For each bacteria indicator and analytical method, four equations were obtained to predict the time required to have a 90% reduction (T90) according irradiance, salinity and temperature parameters. The weighted model RMSE (Root Mean Square Error) calculated for all field experiments showed that quantification obtained with the equations defined by laboratory-based study compared reasonably well with *in-situ* observed quantification (0.4 and 0.2 log by standard culture methods for *E. coli* and *Enterococcus* spp. and 0.6 and 0.3 log by RT-qPCR for *E. coli* and *Enterococcus* spp. respectively). The modeling tool can be used to predict the presence of fecal pollution in marine and fresh waters in combination with either culture based- or rapid molecular methods.

1. Introduction

Fecal indicator bacteria such as *E. coli* and enterococci have been included in most ambient water quality criteria (WHO Guidelines for safe recreational water environments (2003), USEPA Recreational Water Quality Criteria (2012); European Union Directive 2006/7/EC) for mitigating health risks associated with human exposure to water polluted with feces. Agricultural inputs, wild animals, combined sewer overflows, sewage treatment plants, untreated domestic waters, malfunctioning septic systems and industrial wastewater outlets are known to be potential sources of fecal pollution in aquatic systems.

The distribution and abundance of fecal indicator bacteria in recreational waters depend mostly on their input, but also on the intensity of environmental factors and bacterial cell adaptation capacity. Once

introduced into the environment and due to their minor adaptation capacity to environmental conditions, *E. coli* and enterococci cells suffer a sublethal injury (Kapusinski and Mitchell, 1981) and can enter into a dormant, viable but nonculturable state, in which they still maintain some metabolic activity, infective capacity and potential for pathogenicity (Pommepeuy et al., 1996; Oliver, 2005). The “viable but nonculturable” (VBNC) state concept was introduced to describe cells that remain metabolically active but are unable to divide in or on nutritional media that normally support their growth (Roszak and Colwell, 1987). The capacity to form colonies on a solid medium is the first ability that enteric bacteria lose in seawater (Rozen and Belkin, 2001). Among abiotic factors favoring the entrance into VBNC state, the effects of temperature, salinity, and solar radiation are the most pronounced (Canteras et al., 1995; Craig et al., 2004; Kay et al., 2005). With the

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Table 1

Sampling sites and water characteristics at sampling locations, Marbella beach (MB), Uhabia mouth (UM), Uhabia upstream (UU), Saint-Jean-de-Luz Bay (SB), Nivelle port (NP), Nivelle upstream (NU).

Site name	Coordinates	Practical Salinity Units range (PSU)	Nephelometric Turbidity Units range (NTU)	Depth(s) for sampling and <i>in-situ</i> study (m)
Seawater				
MB	43°27'56.37"N 1°35'0.28"O	30–35	0–5	0.5 and 3
SB	43°23'47.25"N 1°40'38.09"O	30–35	0–5	0.5 and 3
Estuarine water				
UM	43°25'54.55"N 1°35'52.19"O	1–35	0–20	0.5
NP	43°23'12.79"N 1°39'56.57"O	27–35	0–20	3
River water				
UU	43°25'57.90"N 1°33'57.52"O	0–0.2	5–60	0.5
NU	43°23'47.25"N 1°40'38.09"O	0–0.2	0–20	0.5

prolonged exposure, particularly in the presence of solar radiation, *E. coli* cells are irreversibly inactivated and they die (Jozić; Šolić, 2017). Although there are many studies in which the effects of the aforementioned factors have been addressed, their simultaneous effects have been poorly investigated.

Knowing the survival rates of fecal indicator bacteria (FIB) is important in evaluating microbial contamination and making appropriate management decision. Existing fate and transport predictive models (eg. Sampaio et al., 2008; Bedri et al., 2016; Thoe et al., 2014; Palazon et al., 2017) account for *E. coli* and enterococci survival by estimating first order decay rates (k) defined as $k = 1/t \cdot \ln(C_0/C_t)$ in units of day^{-1} or hour^{-1} . Decay rates have also been expressed as T90, defined as the time for bacterial concentration to decrease by 90%. While several studies have investigated the decay of FIB in controlled environments, most of them used culture-based methods to established k or T90 values (Noble et al., 2004; Sinton et al., 2007; Ahmed et al., 2014). Moreover, major disagreements among both decay rate constant estimates and the significance of environmental conditions make difficult to forecast how FIB will persist in the environment (Brooks and Field, 2016). Particularly, nearly all of the studies reviewed by Brooks and Field were conducted in artificial environments rather than in an open-system, suggesting possible limitations in the applicability of these estimates in the environment.

In the last decades, culture-independent methods for detecting indicator organisms and microbial source tracking targets have been successfully used, allowing decisions to be made few hours after water sample collection (Haugland et al., 2005). A large Great Lakes study by the US EPA (Wade et al., 2006) showed that enterococci qPCR cell equivalents were related to swimming-associated illnesses in recreational water, heightening qPCR promising use for routine water quality monitoring. However, the persistence of DNA in dead bacteria can be a potential bias for the quantification of bacterial targets (Masters et al., 1994; Deere et al., 1996). The detection of RNA molecules by RT-qPCR (reverse transcription – quantitative polymerase chain reaction), which are much less stable in the environment, is considered as an attractive alternative for sensitive and quantitative detection of FIB and commensal bacteria (Matsuda et al., 2007). In a previous study (Bergeron et al., 2011), significant linear log–log relations between the RT-qPCR and culture method measurements for *E. coli* and *Enterococcus* spp. assays in both fresh and sea waters were shown, allowing the direct use of rRNA-based methods in order to alert about potential pollution in regards with actual water quality criteria for recreational use. As the use of genetic markers for rapid routine analysis increases, it has become important to understand how the decay of these markers is related to traditionally cultured *E. coli* and enterococci.

In this context, this study aimed at establishing T90 formulations related to the three main environmental parameters (temperature, salinity and light) that would be applicable for marine, estuarine and fresh waters, and according to the biological target considered by the analytical methods (culture-based Most Probable Number and RT-qPCR methods). In this study, a laboratory-based microcosm experiment was

undertaken using 24 combinations of the three environmental parameters. To validate the results observed from the laboratory-based microcosm experiments, the persistence of faecal bacteria in both marine and river waters was investigated in field experiments. Decay and hydrodynamic models were constructed and validated to propose prediction tools to forecast the presence of fecal pollution in marine and fresh waters in combination with either culture based- or rapid molecular methods.

2. Material and methods

2.1. Sampling

Different study sites, all located in the South Atlantic Coast in France (Fig. S1, supplementary material), were selected in order to cover a wide range of water type and salinity (sea water, estuarine water and fresh water). Geographical coordinates of the six selected locations and their water quality characteristics are given in Table 1. Previous analysis by culture showed the absence of fecal contamination in the initial samples.

For the laboratory experiments, sea and river water samples were collected in 25 L polyethylene containers and transported within 1 h to the laboratory by minimizing light exposure. For both studies (laboratory and field), sea and river water samples were artificially contaminated by spiking a known quantity of fresh raw wastewater sample collected in Marbella wastewater treatment plant (Biarritz, France) (0.2% vol/vol), in order to obtain a final concentration between 10^3 and 10^5 MPN/100 ml for both *E. coli* and *Enterococcus* spp as determined by Colilert and Enterolert methods (Idexx) within 24 h.

2.2. Laboratory study design

Different experimental conditions corresponding to 24 combinations of 3 environmental parameters were studied as following: two temperature conditions (17 °C and 22 °C); four salinities (33 PSU 17 PSU, 7 PSU and 0 PSU), and three irradiance levels: 0, 13.3 and 63.5 W m^{-2} . Such conditions were designed to cover the typical range of three key environmental parameters (light intensity, water temperature and salinity) of coastal and fresh waters under temperate weather (17 °C and 22 °C corresponding to the mean spring and summer water temperatures, respectively). For the highest and lowest salinities, seawater and freshwater samples from SB and NU sites respectively were used. Samples with intermediate salinities were obtained by mixing the fresh and sea water samples. Irradiance conditions were provided using artificial light (Arcadia Natural Sunlight, 18 W, 600 nm) and measured by ENERCO 411 pyranometer (CIMEL Electronique, France). All the microcosms were set up in 5 L water containers and the experiment was carried out for 5–10 days. During the experiment, 250 ml of samples were taken away from each microcosm for analysis in 500 ml polyethylene bottles, with a periodicity depending on the studied combination (from every 1.5 h for the combination with the

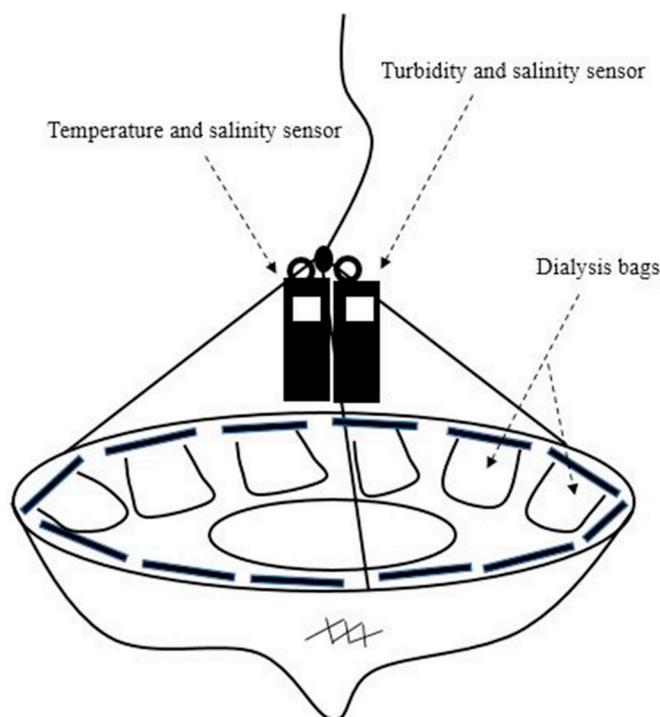


Fig. 1. Diagram of *in-situ* field device.

highest salinity/light and temperature to every 7 h for the dark conditions). A minimum of 15 analyses per condition were performed, with a total of 379 analysis.

2.3. *In-situ* study design

In-situ experiments were conducted during July 2017 in 200 ml volume transparent dialysis bags (Cellu-Sep T3) with a 12 000–14 000 Da molecular weight cutoff containing water from the deployment site mixed with raw sewage of Biarritz wastewater treatment plant (0.2% vol/vol). The pore size of the dialysis bags allowed passage of water and nutrients but prevented passage of bacteria and particles. Experiments were conducted for 5 days. Each day, a minimum of two dialysis bags for duplicate analysis were sampled from each experimental device and poured into sterile flasks. The first day of the experiment started at 08:00 in the morning in order to begin with a day cycle. Samples were stored in cooling conditions and transported to the laboratory within 1 h for being processed.

Dialysis bags were deployed in crab nets and were secured with plastic ties (Fig. 1). The bags were positioned almost horizontally to allow maximal sunlight exposure. We evaluated the impact of sunlight irradiation by placing separate devices at different depths: 0.5 m and 3 m below surface depending on the site. At both depths, turbidity, salinity and temperature were monitored using appropriate sensors (NKE Instrumentation, France). To integrate the continuous measurement of environmental parameters in the predictive models, hourly mean was calculated from the monitoring dataset performed every 5 min or 15 min.

Concerning the irradiance, measurements made by nearby weather stations at water surface were collected. A set of irradiance measures was made by a pyranometer in order to calculate the loss between the light hitting the surface of the water and the light arriving just below. The following equation was then applied for all the sampling points: $I_{\text{belowsurface}} = I_{\text{surface}} * 0.41$. The irradiance attenuation coefficient with the depth was also calculated according to the Secchi disk measures: $k = 1.5/\text{Secchi (m)}$. Finally, the irradiance was calculated with the following equation: $I_{\text{depth}} = I_{\text{surface}} * 0,41 * e^{(-\text{depth}) * k}$

2.4. Standard culture methods (SCM)

For each sample, the ISO 9308-3 & ISO 7899-1 (based on the miniaturized determination of Most Probable Number, MPN) standard methods were used for the enumeration of *E. coli* and *Enterococcus* spp., respectively. A total of four successive dilutions of water samples were initially performed and 200 μl of each dilution were distributed in the 96 wells of the microplate (MUG/*E. coli* or MUD/SF, BioRad) and incubated for 36 h at 44 ± 1 °C. The limit of detection respective to ISO 9308-3 and ISO 7899-1 was therefore 38 MPN/100 ml with an associated confidence interval of 5–271.

2.5. Reverse transcription-quantitative PCR methods

2.5.1. Sample preparation

Analysis was performed with the RNA extraction kit MagSPYD DNA-RNA B (Blue DNA Companion, France). 100 ml of sample was filtered through a 0.45 μm pore-size Hydrophilic Polyvinylidene Fluoride (PVDF) membrane, the filter was then transferred to a 50 ml sterile tube containing 1.8 ml lysis buffer and vortexed for 1 min to release microorganisms retained on the filter. Then, the lysate was transferred to a lysis tube containing glass beads to perform mechanical lysis using a Precellys[®] 24 machine (Bertin Technologies, France). Extraction and purification of total RNA was performed automatically by the KingFisher[™] 96 Robotic processor. This robot was designed to extract and purify total RNA using silica coated paramagnetic beads and chemotropic reagents. All washes were performed automatically using a magnetic rod. DNA was degraded during incubation with DNase. RNA was finally eluted into 500 μl of preservation buffer (preheated at 90 °C). RNA was amplified just after the end of the extraction or stored at -20 °C until used. The maximum storage time for the RNA samples before RT-qPCR quantification is about 24 h.

2.5.2. Quantification by reverse transcription and amplification

16S rRNA specific sequences of *E. coli* and *Enterococcus* spp. were respectively quantified by using GenSpot *E. coli* (*Escherichia coli*) and GenSpot *E. coli* (*Enterococcus*) RTqPCR kits (Blue DNA Companion, France). All sequences are confidential and proprietary and the analytical performances of the assays were previously described by Bergeron et al. (2011). These kits employ the Tth DNA polymerase, a thermostable enzyme with RNA-dependent reverse transcriptase activity and DNA-dependent polymerase activity, allowing the combination of RT and PCR in a single tube reaction. The GenSpot *E. coli*/IPC RT-qPCR kit (Blue DNA Companion, France) is a duplex RT-PCR amplification where an exogenous internal inhibition control constituted by a synthetic RNA fragment detected by specific primers and probe (confidential sequences) is integrated in the pre-amplification mix. A positive amplification of the internal positive control in the same range of the negative control amplification reflects the absence of inhibition.

The amplification reactions were performed in final volume of 20 μl , 5 μl of RNA sample (previously diluted to 1:5) was added to 15 μl of *E. coli* or *Enterococcus* spp. amplification mix. RNA standards were equated to cell quantities by the manufacturer. For performing RT-qPCR quantification, RNA standard provided with the kit was serially diluted and quantified in each run of RT-qPCR.

The amplicons were detected by using the TaqMan MGB probes technology. Analyses were performed using the CFX96 Touch[™] thermocycler from Bio Rad. The thermal cycling program for the CFX96 consisted of an enzymatic activation step of 15 s at 90 °C and a reverse transcription reaction carried out for 20 min at 59 °C, followed by a denaturation step for 30 s and 40 cycles of standard amplification at 92 °C for 5 s and 56 °C for 20 s. Determination of cycle threshold (Ct) was performed automatically by the instrument after manually adjusting the threshold fluorescence values. Using the calibration curve, the software of the real-time PCR equipment calculates the number of equivalent *E. coli* and *Enterococcus* spp. bacteria present in each

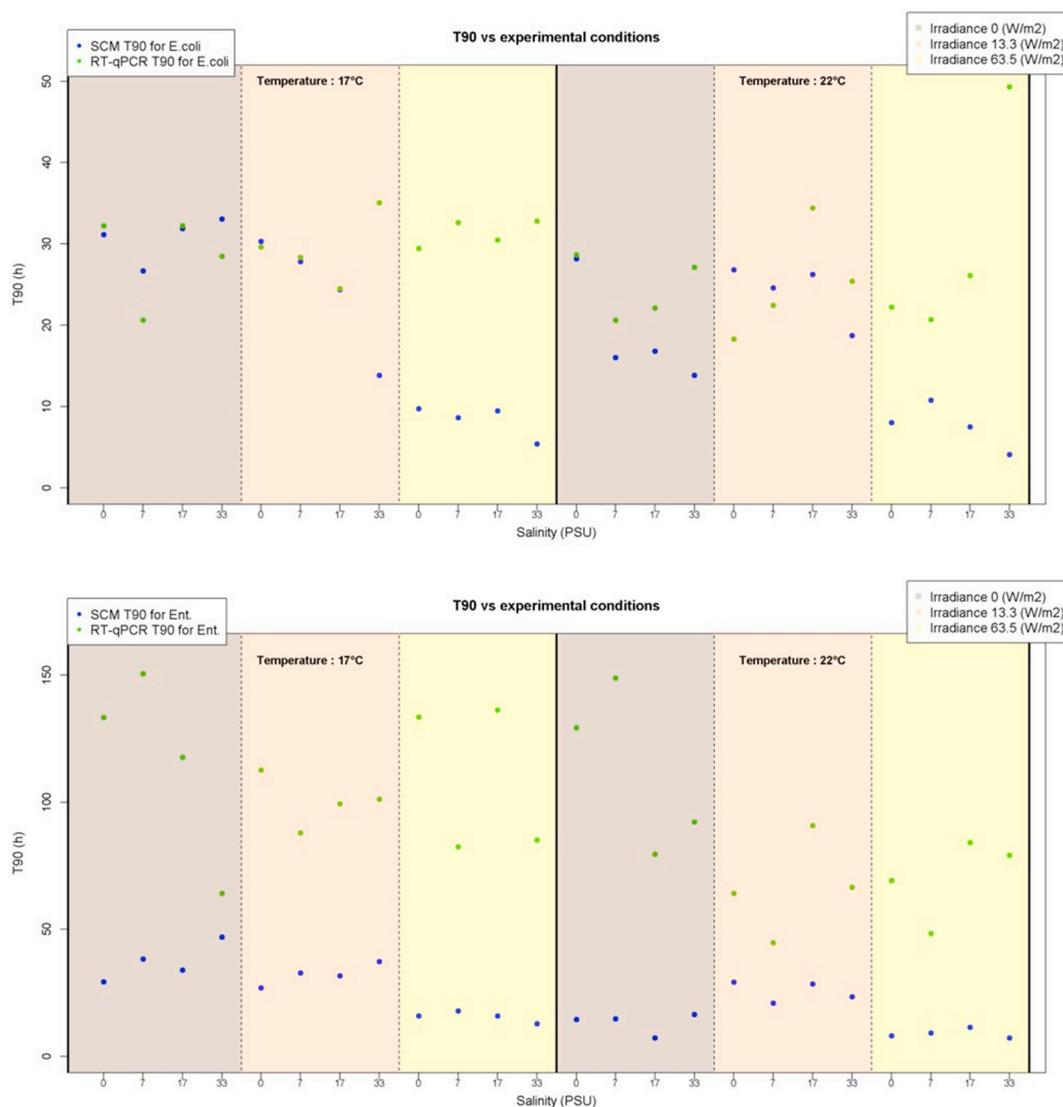


Fig. 2. T90 laboratory results: (A) SCM (Standard Culture Method) and RT-qPCR for *Escherichia coli*; (B) SCM and RT-qPCR for *Enterococcus* spp.

unknown sample by using the Ct values.

2.6. Statistical methods

Statistical tests were undertaken using R statistical package. All microbial concentration data were Ln transformed (natural log) prior to statistical analysis. Bacterial decay rates were assumed to follow a first order decay model according to Chick's Law: $N_t = N_0 e^{-kt}$ where N_t is the number of bacteria at time t , N_0 is number of bacteria at time 0, t is the time (expressed in hours), and k the decay rate over time ($hours^{-1}$). For each experimental conditions, at least 10 time points were used for calculating the corresponding bacterial decay rates. The decay rate (k) is estimated from the slope of the linear regression model fitted to the log-transformed bacterial quantifications. Thus, T90 is calculated as:

$$\ln(0,1 \cdot N_0) = \ln(N_0) - k \cdot T_{90} \Leftrightarrow T_{90} \frac{\ln(0,1)}{-k} = \frac{2,3026}{k}$$

The simple linear regression was applied only to the concentrations higher than the limit of the analytic quantification in order to improve the fit of the decay function. This limit corresponds to the lowest point of the standard curve for Gen-Spot method (250 eq. *E. coli*/100 ml and 16 eq. *Enterococcus* spp./100 ml) for all the studied conditions. For the standard culture methods, the limit of quantification considered is 276

MPN/100 ml, the lowest concentration at which the associated confidence interval is lower than 0.7 log. Below this value, the variability of the MPN estimation is considered too large.

Multiple linear regressions were used to investigate the combined effect of temperature, salinity and solar radiation on changes in the T90 of *E. coli* and *Enterococcus* spp. and to define survival time prediction equations. The RMSE (Root Mean Square Error) was computed between the log of simulated and measured data, for each field experiment, to assess the performance of the formulas.

2.7. Hydrodynamic model

The modeling tool was based on the code MOHID Water Modeling System (Martins et al., 2001; Braunschweig et al., 2004), a 3-D baroclinic, incompressible (Boussinesq), hydrostatic, and free-surface ocean model. MOHID uses a finite volume method to discretize governing equations in a structured Cgrid and a semi-implicit (ADI) temporal algorithm. MOHID Water has been used successfully by the world oceanographic community in complex ocean and coastal applications, e.g. (Malhadas et al., 2009; Riflet et al., 2010), and it is particularly well suited for the study of estuary areas (Sousa et al., 2013). In particular, it has been used for the study of several estuary configurations from the Basque Coast in recent years (Delpey et al., 2014). The robustness of

MOHID allows it to be used for the operational forecasting of the bathing waters quality.

The numerical model used for this work is a 3D model used to investigate dispersion and mixing mechanisms in the bay of Saint Jean de Luz - Ciboure under the combined influence of winds, tide, waves and continental contributions which in particular freshwater inputs from rivers Nivelle and Untxin. The bacteria transport and fate are computed with the water quality model - a dedicated module of MOHID Water - in which the T90 equations are implemented, coupled with the hydrodynamic model. The model provides the spatial fields of all simulated variables, it means their value in each spatial point over time. To date the model is set up for *E. coli* predictions only. The sources of fecal pollution (wastewater treatment plant outlets and rivers) and their average *E. coli* concentrations used as inputs for the model were determined by SCM.

3. Results

3.1. Laboratory-based microcosm study

The duration of the experiments and the frequency of sampling according to the different environmental conditions allowed to observe a significant decay of bacteria. The T90 values and R² values obtained with the Log-linear regression for each analytical method and analyzed bacteria are presented in Fig. 2 and Tables S1, S2, S3 and S4 (supplementary material). For 83% (16 out of 96) of the studied conditions, linearity results were obtained with a confidence interval of 95% (p-value < 0.05). The conditions for which p-value were higher than 0.05 corresponded to rapid decay and an insufficient sampling point number.

For *Escherichia coli* culturable cells, the longest T90 values (T90 > 30 h) were observed for low radiation and temperature conditions (Temperature 17 °C, Irradiance ≤ 13.3 W m⁻²) and for any salinity (except for seawater at 17 °C and 13.3 W m⁻²). The shorter T90 values (T90 ≤ 5 h) were observed for high radiation and salinity conditions. For enterococci, the longest T90 values (up to 47 h) were observed for low radiation and temperature conditions (Temperature 17 °C, Irradiance ≤ 13; 3 W m⁻²), and for any salinity. The shorter T90 values (T90 ≤ 10 h) were observed in condition of high radiation and temperature, for any salinity.

In order to investigate the effect of the three different environmental factors on the inactivation of *Escherichia coli* and *Enterococcus* spp., simple linear regressions were applied to each variable and log of T90 (Figs. S2, S3, S4 and S5, supplementary material). Results indicated that the radiation is the most influent factor affecting *E. coli* and enterococci survival by culture methods (respectively, R² = 0,57 and R² = 0,32, both significant at p < 0.05).

When quantification of molecular markers by RT-qPCR is

considered, *Escherichia coli* decay rates obtained were relatively homogenous and generally comprised between 18 and 49 h⁻¹, without extreme values. Only at the lowest irradiance intensities (below 13.3 W m⁻²) for both temperatures the culturable *E. coli* decayed with the same rate constant as the molecular marker. For low irradiance intensity, the SCM T90 mean is around 24 h, versus 27 h for the RT-qPCR one. For high intensity, there is a significant difference between the means of SCM and RT-qPCR T90, respectively 8 h and 31 h. Simple linear regression results indicated that salinity affects slightly *E. coli* 16SrRNA molecules survival (R² = 0.19, p < 0.05). For enterococci, slow decay rates (T90 ≥ 100 h) were mostly observed for low irradiance and temperature conditions, whereas faster decay rates (T90 between 50 and 100 h) were generally obtained for high irradiance and temperature experiments. The temperature seems to be the most influent factor affecting enterococci survival but without significant correlation (R² = 0,2).

Multiple linear regressions applied to *E. coli* and enterococci (Figs. S6, S7, S8 and S9, supplementary material) showed that the T90 variability could be slightly better explained by the combination of the three environmental factors only for *E. coli* (R² = 0.35 for *E. coli* and R² = 0.20 for *Enterococcus* spp.), but without statistically significant correlation.

The results of *E. coli* and enterococci decay were also compared to a previous set of microcosms-based assays using standard culture methods (Tables S1, S2, S3 and S4, supplementary material). Results showed that discrepancy between decay rates can be observed between independent assays within the same laboratory conditions, especially when the lowest irradiance parameters were applied. Considering all the combined conditions, a total of four equations were obtained to predict the T90: one for each investigated bacteria and analytical method (I = irradiance in W/m², S = salinity in PSU and T = temperature in °C) as follows:

$$T90SCM_ecoli = 318.72 e^{-0.028798 * I} e^{-0.006336 * S} e^{-0.09732 * T}$$

$$T90SCM_entero = 332.13 e^{-0.020477 * I} e^{0.010583 * S} e^{-0.111213 * T}$$

$$T90RT - qPCR_ecoli = 40.88 e^{0.001946 * I} e^{0.007632 * S} e^{-0.028732 * T}$$

$$T90RT - qPCR_entero = 340.53 e^{-0.002315 * I} e^{-0.002815 * S} e^{-0.064508 * T}$$

3.2. In-situ study

3.2.1. Environmental parameters and decay rates

Ambient water quality conditions during the *in-situ* study campaigns were measured every 5 min in the deployed devices (Table 2). Sunshine duration was calculated considering irradiances higher than 120 W m⁻² (Fig. S10, supplementary material). During the first week of July, the sunshine duration and intensity at water surface were higher

Table 2

Field deployment information and physical properties of ambient water during the experimental period. Mean, median (Med), maximum (Max) and minimum (Min) values are presented.

Date & Site name (depth)	Irradiance ^a (W.m ⁻²)			Sunshine duration (h)			Salinity (PSU)			Turbidity (NTU)			Temperature (°C)		
	Mean	Med	Max	Med	Min	Max	Med	Min	Max	Med	Min	Max	Med	Min	Max
July 03 to 07, 2017															
SB (0.5 m)	196	7.7		33.2	30.7	34.7	1.0	0.0	13.0	21.4	21.4	23.5			
SB (3 m)	93			34.9	33.2	35.4	0.2	0.2	17.0	20.4	20.4	21.5			
NP (3 m)	58			33.8	27.2	35.0	2.1	2.1	22.0	20.7	20.7	22.1			
NU (0.5 m)	132			0.1	0.1	0.1	1.0	1.0	17.0	19.7	19.7	21.5			
July 10 to 14, 2017															
MB (0.5 m)	145	4.7		33.7	29.6	35.0	2.4	0.2	22.0	21.0	20.3	22.4			
MB (3 m)	51			35.0	34.3	35.3	2.7	0.5	14.0	21.0	20.3	21.5			
UM (0.5 m)	116			11.0	0.9	34.8	2.5	0.2	22.0	20.3	19.0	22.9			
UU (3 m)	60			0.2	0.0	0.2	14.1	4.5	60.0	18.4	17.7	19.0			

^a Irradiance calculated at the depth studied.

Table 3
E. coli and *Enterococcus* spp. T90 values and decay rates according the analytical methods.

Location	Depth (m)	<i>Escherichia coli</i>					<i>Enterococcus</i> spp.						
		Standard culture method		RT-qPCR			Standard culture method		RT-qPCR				
		T90 (h)	R ²	k (h ⁻¹)	T90 (h)	R ²	k (h ⁻¹)	T90 (h)	R ²	k (h ⁻¹)	T90 (h)	R ²	k (h ⁻¹)
MB	0.5	12	0.76*	-0.19	36	0.69*	-0.06	7	0.93*	-0.32	86	0.56*	-0.03
	3	17	0.86*	-0.13	28	0.84*	-0.08	34	0.8*	-0.07	98	0.61*	-0.02
UM	0.5	40	0.58*	-0.06	54	0.54*	-0.04	29	0.82*	-0.08	335	0.24	-0.01
UU	0.5	47	0.51*	-0.05	49	0.5*	-0.05	44	0.88*	-0.05	118	0.52*	-0.02
SB	0.5	3	0.79*	-0.8	21	0.77*	-0.11	4	0.8	-0.56	51	0.66*	-0.04
	3	11	0.95*	-0.2	32	0.77*	-0.07	9	0.81*	-0.27	66	0.66*	-0.03
NP	3	22	0.9*	-0.11	31	0.64*	-0.08	30	0.79*	-0.08	61	0.83*	-0.04
NU	0.5	69	0.38*	-0.03	40	0.89*	-0.06	29	0.75*	-0.08	54	0.75*	-0.04

*p < 0.05.

than the second week (mean duration and intensity respectively 10 and 25% higher during the 1st week). Results showed that the observed decay rates of the two fecal indicator bacteria were higher (lower T90) for the assays performed at the first week of experiments, for the standard culture method and all environmental conditions, suggesting the dominant effect of solar radiation on the survival of FIB in sea and river water. Indeed, *E. coli* and *Enterococcus* spp. T90 means (on all environmental conditions) were respectively, 26 and 18 h for the first campaign, versus 29 h for both during the second campaign. Without taking into account the NU point (where the irradiance effect could have been limited by the presence of trees and their shades) *E. coli* and *Enterococcus* spp T90 means were respectively 12 and 14 h for the first campaign, versus 23 h for both during the second campaign. Log-linear regression results were significant at p < 0.05 for most of the analyzed conditions (Table 3).

For both fecal indicator bacteria, decay rates observed with standard culture method were higher in sea water and decreased in environmental conditions with lower salinities, establishing a gradient from upstream to downstream. The mean *E. coli* T90 value in sea water was 11 h although it increased from 31 h in estuarine water to 58 h in river water. Similarly to what observed in laboratory-based microcosm assays, longer T90 values were observed for *Enterococcus* spp. than for *E. coli* for both standard culture method (respectively median of 29 h and 19.5 h) and for the RT-qPCR method (76 h and 34 h). The impact of environmental conditions seems higher on the SCM T90 than on the RT-qPCR one. The SCM T90 increases by a factor of 1.5 between sea and estuarine waters and by 0.6 between estuarine and river waters, whereas RT-qPCR T90 one increases by a factor of 1 and then decreases by -0.3. Similarly, the mean SCM T90 for sea waters increases by a factor of 1 between the surface and the mid-depth, whereas the RT-qPCR one does not increase.

3.3. Validation of T90 equations

The four equations defined in controlled conditions experiments were applied to *in-situ* data. Comparisons between estimated concentrations obtained with the application of these equations (simulations) and concentrations observed in the field experiments (data) were carried out and graphically represented. Simulations (predicted values) were obtained by the application of the decay rate formula: $Nt = N0e^{-kt}$ where N0 is the initial concentration of bacteria at time 0 and k is calculated by the utilization of the modeled T90.

An example of the calculated T90 and the predicted *E. coli* and enterococci concentrations calculated by the models in comparison with the observed values is presented in Fig. 3 for both analytical methods (standard culture-based or RT-qPCR methods).

The RMSE calculated for all field measurements showed that the quantification obtained with the equations defined in the laboratory-based microcosm study compared reasonably well with *in-situ* observed

quantification (< 0.6 log) (Table 4). In order to try to reduce errors, SCM model calibrations were performed by adjusting the equations coefficients through the comparison between measured and predicted time series. The more relevant coefficient to be adjusted was the irradiance, according to its dominant influence on culturable bacteria cells. The weighted model RMSE was computed based on the new equations as following.

$$T90SCM_Ecoli = 418.43 e^{-0.035560*0.9*I} prof e^{-0.013677*S} e^{-0.084466*T}$$

$$T90SCM_Enterococcus = 410.05 e^{-0.028791*1.1*I} prof e^{0.004615*S} e^{-0.0916775*T}$$

Results in Table 4 and in Fig. 3(A) and (B) indicated that the weighted models improved the predictions by 0.1 log for both *E. coli* and enterococci for culture-based quantification. For the RT-qPCR method, prediction results were similar whatever the initial or the weighted model as illustrated in Fig. 3(C) and (D).

3.4. Integration of the new T90 equations in the hydrodynamic model

E. coli predictions were examined on the bay of Saint Jean de Luz – Ciboure and river (Untxin), during the time interval from June 5th to 9th, 2018. During that period, a significant rainfall occurred with a total rain of about 4 mm on June 5th and about 23 mm during the night from the 5th to 6th inducing some discharges (combined sewer overflow and wastewater treatment plants) into the environment, mainly on rivers.

The considered T90 equations were calculated from salinity and temperature values as computed by the hydrodynamic model (Fig. S11), and irradiance (Fig. S12) of the nearby weather station (located at around 5 km from the bay of Saint-Jean-de-Luz on the tide gauge of Socoa). Time series were extracted from the modeled fields at three sites: the river mouth (UM) and two beaches named Socoa and Fort Socoa (Fig. S13).

Whatever the considered sites (fresh or sea waters), *E. coli* T90 modeling showed distinct patterns depending on the analytical methods. Especially, the combined positive effects of salinity and irradiance on the T90 RT-qPCR *E. coli* survival can be remarkably observed (Fig. 4). The *E. coli* concentration modeling using the infinite T90 allowed to determine the bacteria decay using T90 as single parameter, highlighting the effect of the dynamics such as dilution, currents, tide and waves effects on the concentration decreases. At the river mouth, where the flow is less turbulent, the T90 was acting predominantly on the *E. coli* concentrations decrease and the difference between both T90 equations appears on the second day (from June 6th in the morning). From that time, the *E. coli* SCM quantification decrease faster than the RT-qPCR one, suggesting the passage into a viable but nonculturable (VBNC) state of bacteria. In contrast, on the bay, the dynamics seemed to strongly impact the decreasing of bacteria whatever the analytical methods. On the Socoa beach, located close to the mouth, the T90

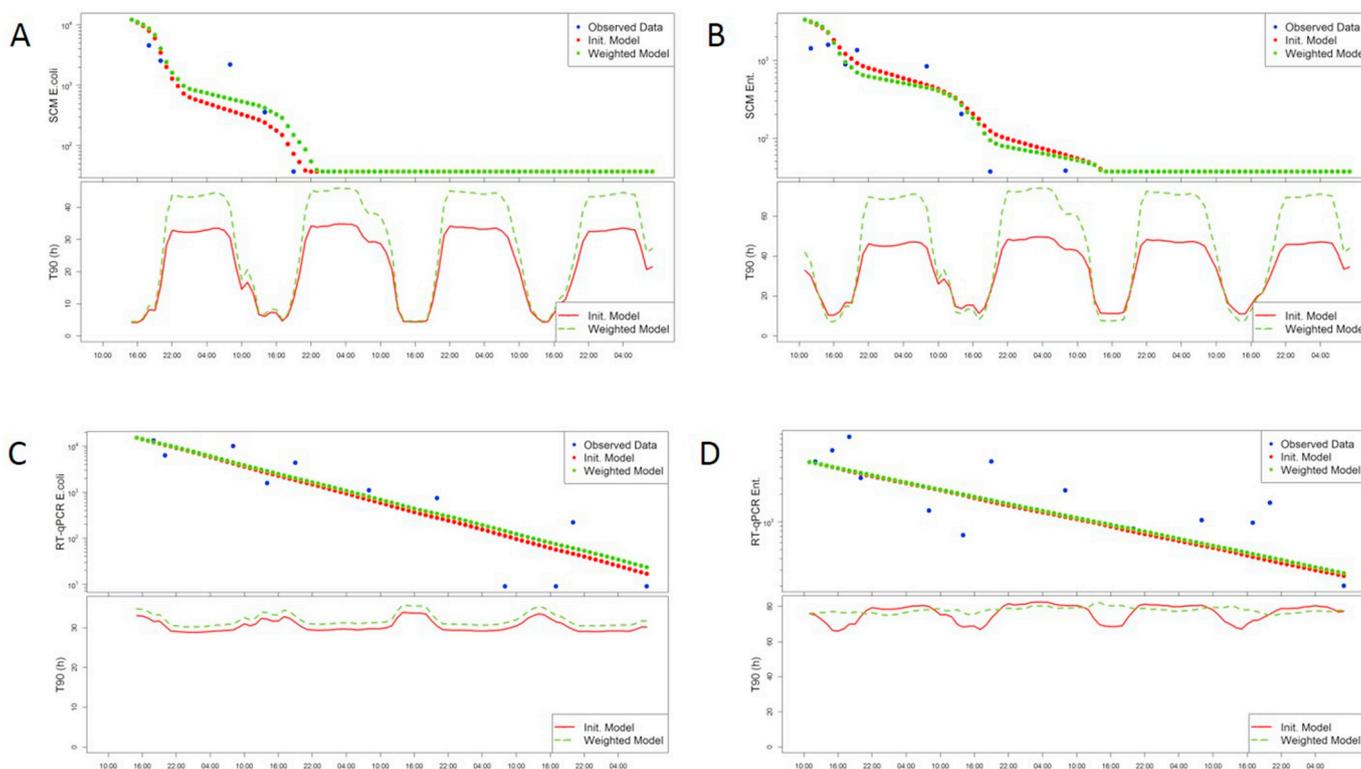


Fig. 3. Comparison of observed and modeled data (with the initial and weighted model) at the Marbella Beach point: (A) SCM quantifications and T90 representation of *Escherichia coli*; (B) SCM quantifications and T90 representation of enterococci; (C) RT-q-PCR quantifications and T90 representation of *Escherichia coli*; (D) RT-q-PCR quantifications and T90 representation of enterococci.

Table 4
Mean error between the log of simulated and measured data.

	Initial model RMSE		Weighted model RMSE	
	<i>Escherichia coli</i>	Enterococcus spp.	<i>Escherichia coli</i>	Enterococcus spp.
Standard culture method	0.5	0.3	0.4	0.2
RT-qPCR	0.6	0.3	0.6	0.3

seemed to influence the *E. coli* decrease from the second day (June 6th, 12pm) whereas on the Fort Socoa point, less impacted by the river plume, the difference between both T90 equations was no longer significant since the dynamics effects had diluted all the bacteria as of the second day (strong dilution the 6th, 12am).

4. Discussion

Laboratory and field studies were carried out to study the *E. coli* and enterococci decay in the coastal and fresh waters in Atlantic French waters, using two analytical methods (culture based- and rRNA based-quantification). In this study, first order decay models were confirmed as the best fitting model for describing all targets behaviors in the laboratory as well as in-situ experimental conditions. In total, 24 laboratory experiment runs were conducted covering the typical range of three key environmental parameters (light intensity, water temperature and salinity) of coastal and fresh waters under temperate weather (17 °C and 22 °C corresponding to the mean spring and summer water temperatures, respectively). The dialysis bags used for the *in-situ* experiments allow water and solutes to exchange between the experiment and the marine and fresh waters, but retain microbial targets and thus represent an open system experimental design as opposed to a closed, batch microcosm design, which physically separates fecal-seeded water from the environmental conditions and can lead to experimental artifacts. However, this design does not allow the determination of the

importance of protist bacterivory that could play in bacteria decay (Lee et al., 2011; Korajkic et al., 2013).

The *E. coli* and enterococci decay rates observed *in-situ* in two consecutive weeks during summer season ranged from 0.03 to 0.80 h⁻¹ by culture methods (corresponding to T90 from 3 to 69 h) and were reasonably represented by the formula derived from laboratory experiments. Although in the recent meta-analysis of fecal indicator bacteria decay studies, results suggested lower k values with a mean value of 0.74 and 0.84 day⁻¹ respectively for *E. coli* and *Enterococcus* spp. (Brooks and Field, 2016), the confidence interval had a much wider range and most of them were derived from only microcosm-based studies. Using similar device for *in-situ* experiments of culturable *E. coli* and enterococci in coastal water, Mattioli et al. (2017) showed k values at surface coastal water and summer season close to those observed in the present study. We also observed that even within the same microcosm experimental conditions, there could be a large amount of variations in the observed decay rate between experimental assays replicated in time, suggesting differences in the physiological states of fecal bacteria cells present in the wastewater sample used as spiking material (Tables S1 and S2, S3 and S4, supplementary material). As observed by different studies (Zhi et al., 2016; Berthe et al., 2013), decay rates of different strains or ecotypes of *E. coli* recovered in sewage systems may be very different due to their adaptations to stress in water. Moreover, different inactivation rates were observed between the FIB depending on the source of the faecal pollution since this was particularly evident

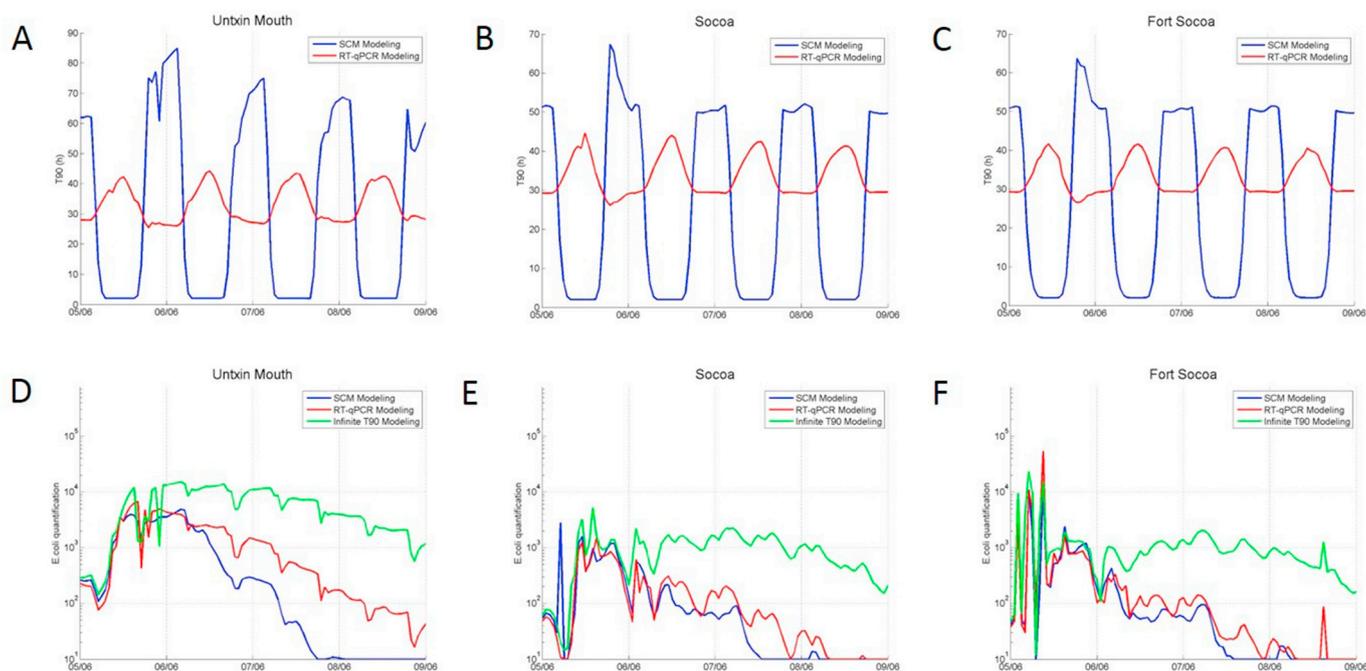


Fig. 4. Modeling of *E. coli* T90 (A, B and C) and *E. coli* concentrations (D, E and F) using RT-qPCR (in red) and SCM (in blue) after a rainfall event. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for pig and ruminant sources for *E. coli* assays (Ballesté et al., 2018).

While sunlight had a large influence on the culture-based enterococci and *E. coli*, it did not influence decay of their respective genetic markers. In our study, low fragments (below to 200 bp) were used to conduct RT-qPCR assays and previous studies showed low UV degradation rates on such short DNA or RNA sequences (Rudi et al., 2010). Moreover, sunlight and salinity are believed to mainly affect the loss of culturability rather than a death or a decay of genetic (rRNA or DNA) material (Servais et al., 2009). Gin and Goh (2013) elucidated the kinetics of transformation of *E. faecalis* into the VBNC state in microcosms and showed decay rates from the culture based method were always higher than the decay rates from PMA-qPCR method (0.002 h^{-1} to 0.24 h^{-1}), implying that cells were able to retain their viability for much longer periods than what is shown by the culture-based method (Gin and Goh, 2013). 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 non-culturable cells are not detected by SCM.

To our knowledge, no existing research has addressed the question of rRNA decay rates although numerous studies based on DNA decay rates (Mattioli et al., 2017; Ballesté et al., 2018; Ahmed et al., 2019). During summer season and at various depths in marine waters, all the k values observed for different human-associated Molecular Source Tracking (MST) DNA-based markers were around 1.5 d^{-1} , corresponding to a T90 around 37 h (Mattioli et al., 2017), as in the same range than *E. coli* rRNA decay rates observed in the present study. Using qPCR methods, the authors also showed no effect of sunlight or season on fecal bacteria DNA marker decay rate. According to the same study, k values determined by Enterococci qPCR assay (US-EPA, 2012) were significantly smaller than the k values of other MST markers. Here a similar trend was observed when comparing *E. coli* and enterococci rRNA decay. The reason for this difference could have to do with divergent grazing pressure with enterococcal species shown as more grazing-resistant than *E. coli* in microcosm assays (Dominguez et al., 2012).

Beach managers are often interested in knowing rapidly the current

state of fecal pollution in the bathing area and how long fecal indicators can persist in the environment at acceptable levels as defined in their respective regulations. The same is true for monitoring and managing the quality of shellfish harvesting waters (Lafforgue et al., 2018). Using predictive modeling as a precursor or alternative to additional qPCR assays will allow to maximize monitoring accuracy and efficiency in the event of rapidly changing conditions. To date, few studies conducted in coastal estuarine or freshwater systems have compared qPCR-based models to culture-based prediction models based on statistical multiple linear regression models (Telech et al., 2009; Byappanahalli et al., 2010; Gonzalez and Noble, 2014). Application of linear regression models indicated that environmental factors explained more of the variability in *Enterococcus* densities measured by SCM than *Enterococcus* densities measured by qPCR. Weighting models by adjusting relevant coefficient corresponding to the irradiance allowed to strengthen the accuracy of predicting models especially when culturable fecal indicator bacteria are monitored. Results also showed that models for both analytical methods did not perform well at predicting occurrences in which water quality levels exceeded a threshold. Indeed, bathing water non-compliance is a complex problem, since it involves many aspects and processes including: catchment management, waste water treatment methods and outfalls, weather (wind, rainfall, irradiance) and hydrodynamic conditions as well as currents, tides and waves, etc. (Huang et al., 2017). Numerical model studies have then been undertaken for predicting fecal indicator organisms in both rivers and coastal waters (de Brauwere et al., 2014), and in some cases a catchment model is used to supply the upper boundary conditions (Bedri et al., 2014). To summarize, such numerical models can be used to: (i) identify the sources, processes and parameters controlling fecal pollution dynamics; (ii) assess the impacts of natural events and human activity on bathing water quality; and (iii) support real-time decision making by providing short-term predictions of fecal bacteria distributions at bathing water and shellfish-harvesting sites.

Usually T90 values used in the modeling systems are based on a fixed average value or simplified for day and night variation only. As showed in this study, decay rates and T90 equations may be included in future water quality models, to assess the fate and transport of microorganisms in different aquatic environments. The integration of the T90

equations in our hydrodynamic model allowed to predict the behavior of *E. coli* concentration and to distinguish the dominant effects from dynamics (dilution, current, tide and wave effects) in seawater systems. However, validation experiments by sampling and analyzing with both quantification methods are required to validate the hypothesis led by the models. For improving prediction, sediment suspension may be included as well as the quantification of wastewater discharges using both culture-based and molecular methods.

In conclusion, our results fill important knowledge gaps about decays of fecal indicator bacteria (*E. coli* and enterococci) markers in marine and fresh waters. Sunlight has the largest influence on the culturable bacteria although it does not influence decay of their respective genetic markers. Using open experimental design for the *in-situ* microcosm study, the quantification obtained from the equations defined by laboratory-based study compared reasonably well with the *in-situ* observed quantification. In addition, decay rates and T90 equations may be included in hydrodynamic models to assess the fate and transport of microorganisms in different aquatic environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijheh.2019.04.011>.

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