Vol. 83: 109–118, 2019
https://doi.org/10.3354/ame01910

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online July 11

Effects of abiotic and biotic factors on *Vibrio harveyi* ATCC 14126^{T} survival dynamics in seawater microcosms

Maite Orruño[1,2,*], Claudia Parada[1], Elixabet Ogayar[1], Vladimir R. Kaberdin[1,2,3], Inés Arana[1,2]

[1]Department of Immunology, Microbiology and Parasitology, Faculty of Science and Technology, University of the Basque Country UPV/EHU, 48340 Leioa, Spain
[2]Research Centre for Experimental Marine Biology and Biotechnology (PIE-UPV/EHU), 48620 Plentzia, Spain
[3]IKERBASQUE, Basque Foundation for Science, 48013 Bilbao, Spain

ABSTRACT: The ability of marine bacteria to adequately respond to environmental abiotic and biotic stressors ensures that they can thrive in diverse aquatic ecosystems. Here, we showed that interaction of *Vibrio harveyi* with natural microbiota can greatly reduce the number of vibrios, and this effect was dependent on temperature. In contrast, the impact of abiotic stress factors was less hostile, primarily affecting *V. harveyi* morphology and physiological state. Although exposure to an oligohaline environment (0.5‰) and nutrient limitation led to a rapid and irreversible loss of *V. harveyi* culturability, higher salinities (15–35.5‰) induced transition to the viable but nonculturable (VBNC) state and cell size reduction in a temperature-dependent manner. Namely, while low temperature (4°C) caused a marked loss of culturability resulting in a dominant VBNC population, higher temperatures (12, 20 and 30°C) affected bacterial culturability to a lesser extent, but resulted in shorter cells after prolonged incubation, especially at 20 and 30°C. In summary, our results define temperature as a key factor that influences *V. harveyi* physiology, morphology and persistence in aquatic systems.

KEY WORDS: *Vibrio harveyi* · Temperature · Predation · Viable but nonculturable

1. INTRODUCTION

The survival and persistence of bacteria in their natural habitats depends not only on their ability to cope with variations in nutrient availability, temperature, salinity, exposure to solar radiation and other stressors (Atlas & Bartha 1998, Pomeroy & Wiebe 2001), but also on the complex interactions that exist among microbial communities (Sherr & Sherr 2002, Hibbing et al. 2010, Sime-Ngando 2014).

Studies of *Vibrio* spp. have revealed that temperature and salinity are the most significant abiotic factors that determine the fate of these bacteria in their natural habitats (Johnson 2013, Takemura et al. 2014, Di et al. 2017). Moreover, the seasonal dynamics of vibrio populations is mainly modulated by temperature (Takemura et al. 2014, Di et al. 2017, Girard et al. 2017). While low temperature reduces *Vibrio* culturability and induces its entry into a viable but nonculturable (VBNC) state (Asakura et al. 2007, Nowakowska & Oliver 2013, Abia et al. 2016), temperature upshifts increase the abundance of *Vibrio* populations (Vezzulli et al. 2013). In addition, the abundance of *Vibrio* spp. in coastal areas is also affected by salinity. Vibrios are usually regarded as moderately halophilic bacteria, and the vast majority of them require Na^{+} for growth and survival in aquatic systems (Soto et al. 2010). However, high

*Corresponding author: maite.orruno@ehu.es

salinities found in the open ocean (typically ca. 35‰) can inhibit *Vibrio* spp. growth (Oliver 2015, Davis et al. 2017). Likewise, low concentrations of salt have a negative effect on the capacity of many vibrios to preserve culturability (Eguchi et al. 2000, Armada et al. 2003) and can markedly shorten the time required for these bacteria to enter into the VBNC state (Wong & Wang 2004), a phenotype that can be acquired by some bacteria facing adverse conditions. In fact, various members of the *Vibrio* genus were extensively used to reveal the main features of this state (Xu et al. 1982, Oliver 2015, 2016). It has been demonstrated that, once the VBNC phenotype is acquired, the cells lose the ability to grow on standard media commonly used to culture them under laboratory conditions (Roszak & Colwell 1987, Oliver 2016). Nevertheless, the VBNC state is reversible and, when environmental conditions become favourable, the VBNC cells can recover and resume growth (resuscitation process) (Oliver 2010, Ramamurthy et al. 2014).

Among biotic stressors, bacteriophages and heterotrophic protists represent the key factors controlling the diversity and abundance of marine bacteria (Fuhrman 1999, Wommack & Colwell 2000, Sun et al. 2013). Besides the significant contribution of grazing by bacteriovorous protist to the mortality of vibrios in aquatic environments (Hahn & Höfle 2001, Sherr & Sherr 2002), it also represents the major selective force driving the evolution of cell adaptation mechanisms (Matz & Kjelleberg 2005). One of them is based on the capacity of microbial communities to survive by attachment to diverse surfaces and subsequent biofilm formation (Chávez-Dozal et al. 2013, Lutz et al. 2013). Takemura et al. (2014) have shown that, although most populations display various preferences to adhere to different organic surfaces, including zoo- and phytoplankton, some *Vibrio* spp. successfully thrive in a free-living state. Nevertheless, planktonic vibrios are scarce and their low abundance in marine environments is likely due to the predatory activity of flagellates (Beardsley et al. 2003), whose presence and lifestyle are also influenced by variations in environmental parameters such as temperature (Atkinson et al. 2003).

V. harveyi has recently emerged as an important pathogen of both marine invertebrates and vertebrates (Austin & Zhang 2006, Haldar et al. 2010), negatively affecting their health in a temperature-dependent manner (Fukui et al. 2010, Cardinaud et al. 2014). Despite recent studies addressing the effect of temperature on adaptation of *V. harveyi* in seawater microcosms (Sun et al. 2008, Montánchez et al. 2014, Kaberdin et al. 2015, Parada et al. 2016), little is known about the specific roles of other major stress factors.

The main goal of the present work was to deepen our knowledge about the contribution of temperature, salinity and protist community to *V. harveyi* persistence and survival under nutrient-limiting conditions.

2. MATERIALS AND METHODS

2.1. *Vibrio harveyi* strains and inocula preparation

Two *Vibrio harveyi* strains, *V. harveyi* strain ATCC 14126^T (hereinafter *V. harveyi*) and its isogenic variant tagged with green fluorescent protein (GFP) (hereinafter *gfp*-tagged *V. harveyi*) were used in this study. The *gfp*-tagged strain was constructed by triparental conjugation between the strains *Escherichia coli* DH5 *pir*, *E. coli* CC118 *pir* (that carries the donor and helper plasmids pVSV102 and pEVS104, respectively) and *V. harveyi* (kanamycin and nalidixic acid sensitive; Clinical and Laboratory Standard Institute 2017). Both *E. coli* strains were kindly provided by Eric V. Stabb (University of Georgia, Athens, GA; Dunn et al. 2006). The conjugants were selected based on their resistance to kanamycin (100 µg ml^{-1}) and emission of green fluorescence. The resulting *gfp*-tagged *V. harveyi* exhibited similar growth rate (0.64 ± 0.01 h^{-1}) as the non-tagged parental strain (0.62 ± 0.01 h^{-1}). The strains were stored at −80°C using the Microbank™ bacterial preservation system (Pro-Lab Diagnostics).

For inocula preparation, *V. harveyi* cells were cultured aerobically in marine broth (MB; Panreac AppliChem) in the absence or presence of kanamycin (100 µg ml^{-1}) and incubated at 26°C with shaking (120 rpm) until they reached the stationary phase (24 h). Afterwards, the cells were harvested by centrifugation (4000 × *g*, 4°C, 20 min), washed 3 times with sterile saline solution (1.94% NaCl, w/v) and, finally, suspended in it.

2.2. Survival experiments and resuscitation procedure

All the glass flasks used for performing survival experiments with *V. harveyi* cultures were cleaned with H_2SO_4 (96% v/v) beforehand, rinsed with deionized water and kept at 250°C for 24 h to remove residual organic compounds.

For survival assays in the absence of natural seawater microbiota, *V. harveyi* populations were incubated in sterile natural seawater (NSW) with salinity 35–37‰ from Port of Armintza in the North of Spain (43° 26′ 24″ N, 2° 54′ 24″ W). Namely, Erlenmeyer flasks containing 200 ml filtered (by means of sequential filtration through 8, 0.8, 0.45 and 0.22 µm pore-size filters; Millipore) and autoclaved seawater were inoculated to reach a density of 10^8 cells ml^{-1} and incubated at 4, 12, 20 and 30°C with shaking (120 rpm) in darkness. In addition, artificial seawater (ASW) (Sea Salts, Sigma) was used to prepare media with various salinities. In this case, *V. harveyi* suspensions were also incubated at 20°C in darkness by using Erlenmeyer flasks filled with 200 ml of undiluted (35.5‰ salinity) and diluted (30, 15 and 0.5‰ salinity) sterile ASW. The salinity of NSW, undiluted ASW and its diluted variants was determined by using a salinometer (ProfiLine Cond 197i WTW; ICT). Samples that originated from 3 biological replicates were used for determining bacterial counts (total, viable and culturable fractions), monitoring the entry of cells into the VBNC state and measuring the cellular size.

Populations of the survival experiments that reached the point when the culturable fraction decreased below the limit of detection (i.e. <0.33 CFU ml^{-1}), and therefore the cells with the VBNC phenotype were dominant, were further processed to determine the ability of nonculturable cells to resuscitate. The resuscitation experiments were performed according to the procedure previously described by Parada et al. (2016). Briefly, the resuscitation was carried out in series of 5 tubes containing 9.9 ml of sterile NSW supplemented with nalidixic acid (4 µg ml^{-1}) to avoid DNA replication. Tubes were inoculated with 0.1 ml of VBNC populations of *V. harveyi* (<0.33 CFU ml^{-1}) obtained as described above and incubated at 20 and 26°C for 7 d. Then, the total and culturable cells were determined as described in Section 2.3.

The effect of autochthonous microbial populations of seawater on *V. harveyi* fate and survival was assessed by maintaining *gfp*-tagged *V. harveyi* populations in nonsterile NSW, in which the *gfp*-tagged *V. harveyi* cells were clearly distinguished from the rest of the microbial community due to their fluorescence. In these experiments, the initial *gfp*-tagged *V. harveyi* density was about 10^6 cells ml^{-1}, which was close to the density of total indigenous bacteria present in the seawater used (see below). These experiments were carried out at 4, 12, 20 and 30°C. Periodically, samples were withdrawn for determining the total and culturable bacteria (indigenous bacteria and inoculated *gfp*-tagged *V. harveyi*) and the number of protozoa, as described in Section 2.3.

All experiments were performed in triplicate. The values presented in data sets are the means of 3 experiments, and the standard deviations between replicates were less than 12%. The differences between the means were calculated by 1-way ANOVA. Probability values ≤ 0.05 were considered significant.

2.3. Microbial counts

The total number of bacteria (TNB) was determined after cell staining with acridine orange according to the procedure described by Hobbie et al. (1977). Direct enumeration of *gfp*-tagged *V. harveyi* fluorescence cells was carried out by epifluorescence microscopy of unstained samples. Bacteria in at least 20 randomly selected microscope fields containing 20–30 cells $field^{-1}$ were counted.

In the experiments carried out to determine the influence of NSW protozoa communities on the survival of *gfp*-tagged *V. harveyi*, the actual number of seawater bacteria was estimated by subtracting the number of *gfp*-tagged *V. harveyi* from the TNB determined in samples stained with acridine orange.

Bacteria with intact cytoplasmic membranes (MEMB+) were counted with the Live/Dead Bac Light™ kit (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's specifications. Given the impossibility of distinguishing the green fluorescence attributable to *gfp* gene expression from the fluorescence emitted by the fluorochrome SYTO 9 (Live/Dead BacLight™ kit), MEMB+ cells were not counted in the experiments carried out with NSW microbiota.

To determine the number of culturable *V. harveyi* cells (CFU), aliquots of cell suspensions were plated on Marine Agar (MA; Panreac AppliChem) followed by their incubation for 24 h at 26°C. In the experiments carried out in the presence of the seawater microbiota, the *gfp*-tagged *V. harveyi* cells were enumerated by plating on Marine Agar supplemented with kanamycin (100 µg ml^{-1}; Sigma-Aldrich) and the fluorescence emission of the colonies was verified under illumination provided by a UV-A lamp (UVGL-58). The total number of chemoorganotrophic culturable bacteria was determined by counting the nonfluorescent colonies that were formed on Marine Agar in the absence of kanamycin after incubation for 48 h at 26°C.

The method described by Sherr et al. (1988) was used to determine the density of flagellated and ciliated protozoa.

2.4. Estimation of *V. harveyi* size

The length variations of *V. harveyi* cells during their survival were estimated via image analysis of epifluorescence preparations (Massana et al. 1997) by using an image analysis system, which included a video camera of high resolution (Hamamatsu 2400; Hamamatsu Photonics). Digitized images of microscopic fields were analyzed by Scion Image 1.62[a] software. The mean value (x) and the corresponding standard deviation (SD) were established in the course of each survival experiment. Moreover, the mean value which defined the size of the cells in the initial inoculum (x_0), was used to establish 3 ranges of cell size ($\leq x_0$ − SD, $> x_0$ − SD to $\leq x_0$ + SD, $> x_0$ + SD), subsequently used to present the time-dependent changes of cell size in *V. harveyi* populations (Parada et al. 2016).

3. RESULTS

3.1. Survival of *V. harveyi* under starvation at different temperatures

The incubation temperatures (4, 12, 20 and 30°C) used in the present study corresponded to the average temperatures of the sea surface water for the cold (12°C) and warm (20°C) seasons in our coastal area (Goikoetxea et al. 2009), as well as those that were close to the upper (30°C) and lower (4°C) limits of sea surface temperatures used in previous studies (Levitus et al. 2000, Sun et al. 2008, Parada et al. 2016).

Fig. 1 shows results obtained during survival assays of *Vibrio harveyi* ATCC 14126^T performed in sterile NSW at different temperatures (i.e. 4, 12, 20 and 30°C) in the absence of natural microbiota. In all cases, the number of total and MEMB+ *Vibrio* cells remained almost unchanged throughout the incubation time. Nevertheless, the number of CFU decreased by >1 log cells (≤0.05) at the end of the incubation time at 4, 12 and 30°C, and was most affected at the lowest temperature studied. Moreover, these results indicated that >99.9% of cells likely entered the VBNC state at 4°C.

In addition, there was a continuous reduction of cell size observed over the entire temperature range. Specifically, although at the beginning of the assays the average length of the cells was close to 1.15 (±0.29) µm, the reduction of cell size (Fig. 1) led to a considerable increase in the percentage of cells with a length equal to or shorter than 0.9 µm (Table 1). The beginning and magnitude of this process were temperature-dependent. At lower temperatures

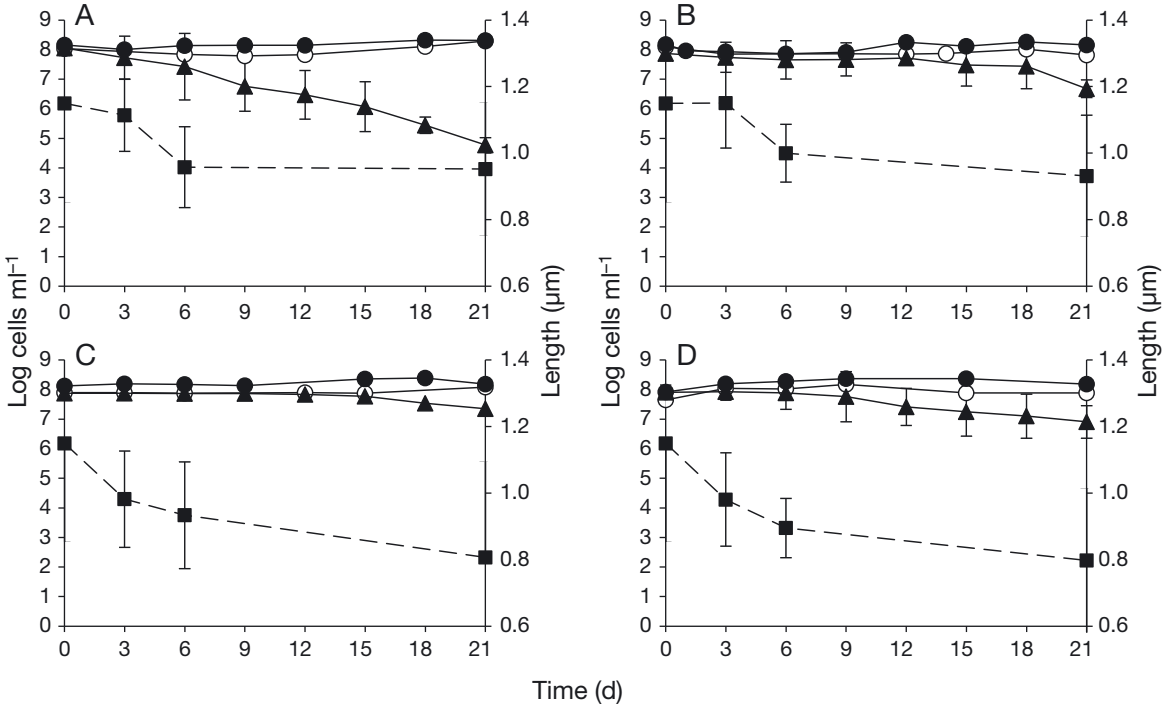

Fig. 1. Survival of *Vibrio harveyi* ATCC 14126^T populations maintained in sterile natural seawater incubated at (A) 4°C, (B) 12°C, (C) 20°C and (D) 30°C showing variations of total (●), with intact membrane (○), culturable bacteria in Marine Agar (▲) and cellular length (■). Data are mean (±SD) values from 3 independent experiments

Table 1. Variation in cell size distribution (mean ± SD) obtained after 21 d of incubation of *Vibrio harveyi* ATCC 14126^T in sterile natural seawater incubated at different temperatures and in sterile diluted (0.5, 15 and 30‰) and non-diluted (35.5‰) artificial seawater

Size range (µm)	Initial	Temperature (°C)				Salinity (‰)			
		4	12	20	30	0.5	15	30	35.5
≤0.9	13.0 (± 1.3)	42.5 (± 4.6)	51.0 (± 5.9)	58.0 (± 6.9)	65.4 (± 6.7)	21.0 (± 1.9)	72.4 (± 8.1)	87.3 (± 9.2)	89.2 (± 10.4)
>0.9 to ≤1.4	73.0 (± 4.9)	50.0 (± 9.7)	47.5 (± 10.1)	41.5 (± 9.3)	32.4 (± 7.4)	55.8 (± 9.8)	26.8 (± 2.0)	12.7 (± 0.8)	10.8 (± 0.1)
>1.4	14.0 (± 3.1)	7.5 (± 2.1)	1.5 (± 0.2)	0.5 (± 0.1)	2.2 (± 0.3)	23.2 (± 2,1)	0.8 (± 0.1)	0.0	0.0

(4 and 12°C), cells maintained their initial size for a few days before they started undergoing fast reduction (between Days 3 and 6) and then the process was slowed down so that the average length of approximately 0.94 (±0.19) µm was reached by the end of the incubation time (i.e. after 21 d). For populations incubated at higher temperatures (20 and 30°C), the size reduction began earlier, resulting in an average length of the cells of 0.98 (±0.14) µm after 3 d and 0.80 (±0.21) µm at the end of the incubation period. In fact, the percentage of short cells (i.e. ≤0.9 µm) was 13.0% at the beginning of the experiments and reached much higher values at the end of the incubation period for populations maintained at 20 and 30°C (58.0 and 65.4%, respectively) than for those exposed to 4 and 12°C (42.5 and 51.0%, respectively) (Table 1).

3.2. Survival of *V. harveyi* under starvation at different salinities

The range of salinities was chosen to simulate oligo- (0.5‰), meso- (15‰), poly- (30‰) and euryhaline (35.5‰) aquatic environments. The effect of salinity on survival of *V. harveyi* at 20°C is shown in Fig. 2. Throughout the survival process, no significant changes in total cell counts were observed over

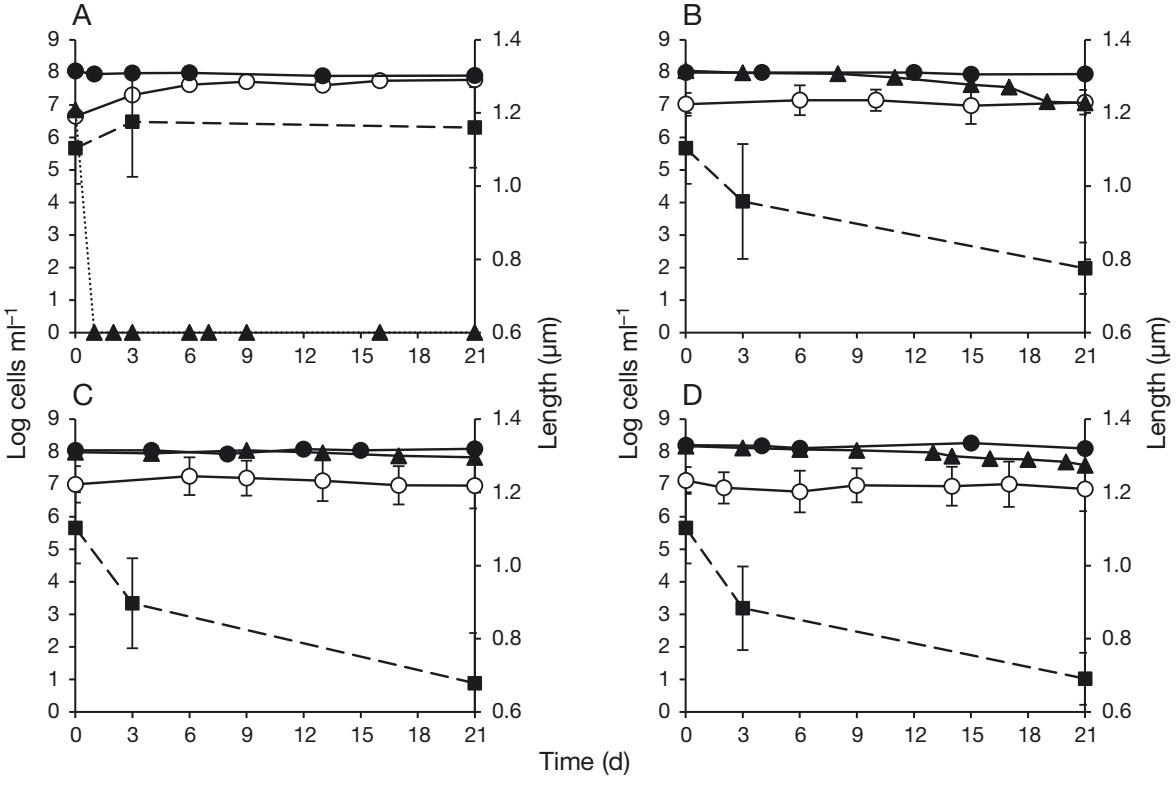

Fig. 2. Survival of *Vibrio harveyi* ATCC 14126^T exposed to different salinities: (A) 0.5‰, (B) 15.0‰, (C) 30.0‰ and (D) 35.5‰ in sterile artificial seawater (absence of nutrients) at 20°C, and variations of total (●), with intact membrane (○), culturable bacteria in Marine Agar (▲) and cellular length (■). Dotted line in (A) indicates culturable cell counts below detection limit of the enumeration method. Data are mean (±SD) values from 3 independent experiments

the entire range of salinities. Moreover, the count of colonies obtained by spreading cell suspensions on MA did not reveal a significant loss of culturability in experiments carried out at higher salinities (30 and 35.5‰). In contrast, the culturability declined from 10^8 to 10^7 CFU ml^{-1} during the first 10 min of incubation and reached undetectable levels (<0.33 CFU ml^{-1}) within 24 h in oligohaline microcosms (0.5‰). When cells were maintained in mesohaline media (15‰), their culturability declined by 90% after 21 d.

For populations exposed to different salinities, the counts of cells with intact membranes were clearly lower (about 1 log cells) than those of culturable cells at the beginning of the study (Fig. 2). These values remained constant for 21 d (Fig. 2B–D) or even increased under hypoosmotic/oligohaline conditions (0.5‰) (Fig. 2A). The latter indicates that hypoosmotic conditions per se decrease *V. harveyi* membrane permeability, and therefore, the data obtained under these conditions cannot directly be related to cell viability.

Resuscitation experiments were performed with the nonculturable populations of *V. harveyi* obtained after 24 h in low salinity (0.5‰) microcosms. Attempts to resuscitate these cells upon incubation in sterile NSW at 20 and 26°C for 7 d did not yield any positive results (data not shown).

As for morphological changes, the cells incubated at salinities in the range of 15–35.5‰ reduced their length. Moreover, the percentages of reduced rods (≤0.9 µm) were higher (>72.0%) than those previously obtained for populations maintained in sterile NSW (58.0%) (Table 1) at the same temperature. In contrast, *Vibrio* populations maintained in hypoosmotic/oligohaline conditions (0.5‰) (Fig. 2A) underwent a slight increase in size.

3.3. Influence of biotic factors on *gfp*-tagged *V. harveyi* survival

Upon exposure to autochthonous microbiota initially present in NSW, total and culturable counts of *gfp*-tagged *V. harveyi* declined at all temperatures studied (Fig. 3). In other words, the total number of *gfp*-tagged *V. harveyi* cells declined by 4 log cells by

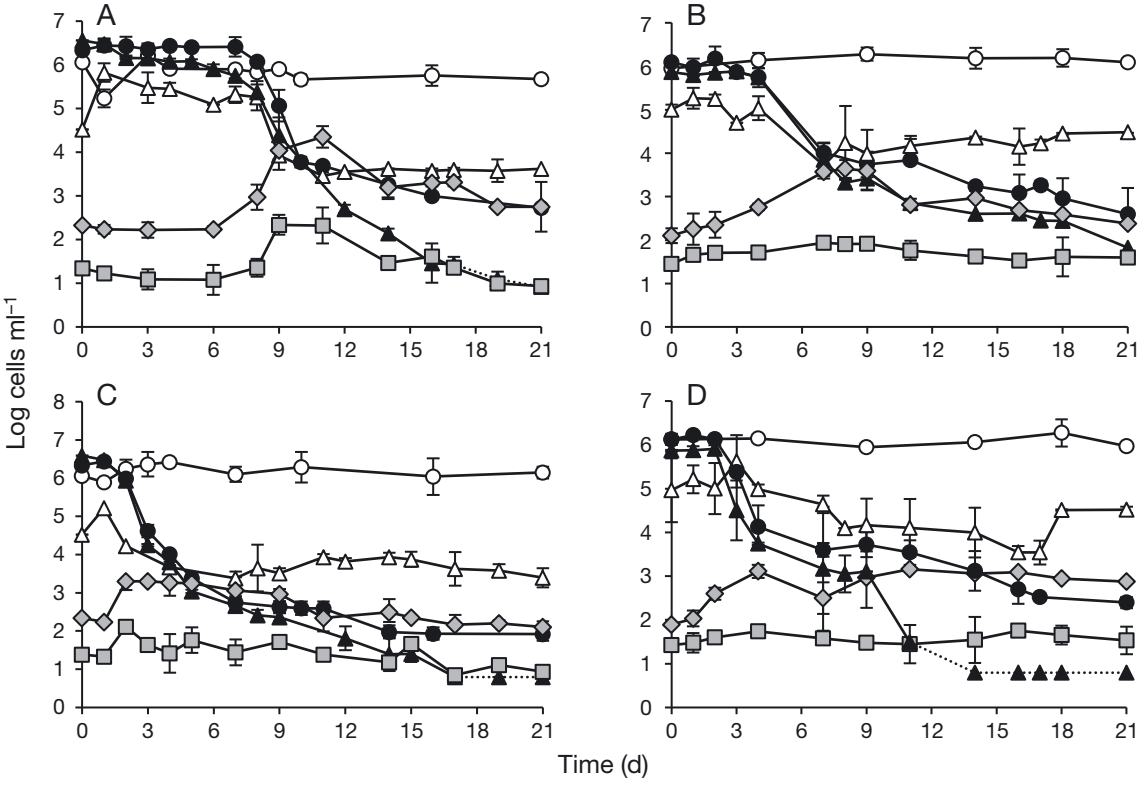

Fig. 3. Survival of green fluorescent protein (*gfp*)-tagged *Vibrio harveyi* populations in the presence of seawater microbiota at (A) 4°C, (B) 12°C, (C) 20°C and (D) 30°C. Variations of seawater-indigenous total bacteria (○), *gfp*-tagged *V. harveyi* (●), chemoorganotrophic culturable bacteria (△), culturable *gfp*-tagged *V. harveyi* (▲), total number of flagellated (◇) and ciliated (□) protozoa. Dotted lines: culturable cell counts below detection limit of the enumeration method. The data are mean (±SD) values from 3 independent experiments

the end of the experiment and the culturability reduced by ≥5 log cells, below the detection limit in the assays performed at 4, 20 and 30°C. The differences found between total and culturable counts at the end of incubation period indicated that part of the population probably entered the VBNC state, thus mimicking the *V. harveyi* response observed in the absence of natural microbiota (Fig. 1).

Following a common predator–prey interaction, a decrease of *gfp*-tagged *V. harveyi* population was accompanied by a concomitant increase of the number of protozoa. Moreover, the predator–prey interaction was temperature-dependent. Namely, the predatory response was faster at higher temperatures and it took protozoa only 2–3 d to start greatly reducing *gfp*-tagged bacteria populations at 20 and 30°C, whereas similar effects could be observed at 12 and 4°C only after 5–6 and 8 d, respectively. Nevertheless, we cannot exclude that a part of the bacterial population was likely eliminated due to infection with bacteriophages, although their potential impact has not been determined in this work.

Compared to *gfp*-tagged *V. harveyi*, indigenous bacteria showed a clearly different behaviour. While total seawater bacteria counts remained unaltered, the culturability of chemoorganothophic bacteria fluctuated and was reduced by the end of the assays but not greater than 1 log cells. Moreover, the initial decreases in number of chemoorganothophic and *gfp*-tagged *V. harveyi* cells were inversely related to the observed increase in the protozoan community. Nevertheless, the counts of culturable chemoorganotrophic bacteria were 3–4 log cells higher than those of culturable *gfp*-tagged *V. harveyi* at the end of the assays. Therefore, the results suggested that a small fraction of the natural bacterial population present in the seawater also underwent predation by protozoa or bacteriophages, although to a lesser extent than the *gfp*-tagged *V. harveyi.*

4. DISCUSSION

Vibrio species inhabit diverse aquatic systems, in which fluctuations in environmental parameters (e.g. variations in salinity within estuarine areas or temperature changes at the sea surface) can frequently happen, thereby affecting the variety, dynamics and composition of these ecosystems (Eiler et al. 2006, Urakawa & Rivera 2006).

Here, we have demonstrated that biotic and abiotic factors can differentially affect the survival and physiological state of *V. harveyi.* While exposure to predators (and likely bacteriophages) can nearly completely and irreversibly eliminate *V. harveyi* populations, the effect of abiotic stress factors could be reversible and primarily affects cell morphology and physiology.

Among the abiotic factors tested in this work, only exposure to hypotonic conditions (0.5‰) had a devastating effect on *V. harveyi*, consequently leading to a rapid (and apparently irreversible/irreparable) loss of culturability accompanied by a slight increase in cell size. This morphological change was likely caused by a decrease in external osmolarity that altered cellular turgor, thereby causing water uptake (Booth & Blount 2012). In other words, the osmotic influx of water apparently stretched the elastic cell wall and the outer membrane (Rowe et al. 2013), thus counteracting cell size reduction frequently observed during *V. harveyi* adaptation to starvation (Kaberdin et al. 2015). Although the osmotic disequilibrium provoked by low salinity could have a deleterious and irreversible effect on *V. harveyi* cells, our data revealed that, in the absence of natural microbiota, *V. harveyi* can successfully deal with moderate fluctuations in temperature and salinity.

In the range of temperature studied (4–30°C), the lowest (i.e. 4°C) led to a great increase in the number of VBNC cells (>99.9% of cells were nonculturable after 21 d of exposure), compared to the moderate and time-delayed decrease in culturability observed for populations maintained at 12 or 30°C. These results are consistent with those obtained by other authors for different *Vibrio* spp. maintained in sterile seawater (Kaspar & Tamplin 1993, Gauthier 2000, Coutard et al. 2007, Oliver 2015). Although sterilization (by filtration and autoclaving) of NSW could potentially lead to a slight change in nutrient content (Ammerman et al. 1984), it likely had only a minor impact on *V. harveyi*, and the survival patterns exhibited by populations maintained in sterile NSW were primarily dependent on temperature.

During the maintenance of *Vibrio* populations in sterile NSW (i.e. under limitation of nutrients), the length of the cells gradually decreased, and this process was temperature-dependent. Namely, the populations exposed to 20 and 30°C underwent the greatest reduction in length so that the fraction of small cells (≤0.9 µm) became dominant. Similar changes in length were observed for *Vibrio* populations maintained in sterile ASW at salinities ranging from 15–35.5‰. Some authors have proposed that reduction of cell size could be related to cell entry into the VBNC state (Falcioni et al. 2008, Sun et al. 2008); however, our recent studies (Montánchez et al. 2014,

Kaberdin et al. 2015, Parada et al. 2016) indicate that, at least for *V. harveyi*, this morphological change likely represents an early response to starvation.

Regarding the dynamics of phenotypic changes, reduction of cell size was more pronounced for populations maintained in the absence of nutrients (ASW in the range of 15–35.5‰), compared to cells incubated in NSW at the same temperature (20°C). This finding suggests that *Vibrio* size reduction is a process controlled by nutrient availability and temperature.

Unlike temperature, salinities similar to those of marine (30–35‰) or estuarine (15‰) waters did not seem to play a critical role in controlling *V. harveyi* survival. Interestingly, most of the cells maintained in ASW at these salinities possessed red fluorescence after staining with the Live/Dead BacLight™ kit. Although red fluorescence could be indicative of damaged cytoplasmic membranes, the culturablility of *V. harveyi* cells was largely unaffected. In other words, it seems likely that an increase in membrane permeability (i.e. red fluorescence) mainly represents an adaptation change that enhances cell fitness without any significant effect on cell viability. The capacity to preserve viability while showing permeability for propidium ions has also been reported for other Gram-negative and Gram-positive bacteria (Yang et al. 2015, Kirchhoff & Cypionka 2017). The stress-induced permeability of the cell membrane appears to be a survival or evolutionary strategy of cells exposed to harsh environments, as partly permeable membranes can facilitate communication between cells and the environment (Yang et al. 2015). Therefore, although the use of the Live/Dead BacLight™ kit can reveal changes in membrane permeability, it may not be appropriate for assessing the viability of cells in ASW. Although *V. harveyi* can preserve cell viability at moderate and high salinities, the cells that were maintained under oligohaline conditions seemed to undergo irreversible injury, even though their cytoplasmic membranes were apparently intact and the cells retained turgor. Moreover, the deleterious effect of low salinity was different from that found in NSW microcosms, in which VBNC populations obtained after prolonged incubation at low temperatures still preserved their capacity to resuscitate (Parada et al. 2016).

Bacterial survival in aquatic environments is greatly influenced by various biotic factors, including protozoa (especially nanoflagellates and ciliates; Sherr & Sherr 2002, Urakawa & Rivera 2006) and bacteriophages (Jacquet et al. 2010). We found that *V. harveyi* was a preferred prey of protozoans, primarily flagellates, and that the protozoa-mediated grazing greatly reduced the *V. harveyi* population up to a threshold level, known to be dependent on flagellate type and temperature (Ishigaki & Sleigh 2001, Delaney 2003, Beveridge et al. 2010). The simultaneous increases in flagellate protozoa populations and decreases in *V. harveyi* abundance varied with temperature and were consistent with the expected role of protozoa as one of the main factors controlling *V. harveyi* populations. Thus, our findings suggest that, besides its great impact on the physiology of *V. harveyi*, temperature also regulates its grazing by marine microbiota, thus affecting *Vibrio* mortality in aquatic ecosystems.

In summary, our data provide new insights regarding the impact of temperature, salinity and marine microbiota on *V. harveyi* persistence in its natural habitat. Nevertheless, the above findings cannot satisfactorily explain the observed seasonal distribution of *Vibrio* spp. and suggest that other factors might also contribute to *Vibrio* adaptation and survival. These additional factors are likely related to bacteriophage activity or biofilm formation. The impact of vibrio-specific phages on the seasonal abundance of vibrios has recently been studied by using *V. cholera*. Faruque et al. (2005) found an inverse correlation between the presence of vibriophages affecting the seasonal variability of *Vibrio* populations and occurrence of viable *V. cholerae* cells. In addition, biofilm formation has been suggested as an alternative survival strategy adopted by vibrios (Sun et al. 2013, 2015) to provide adequate protection in adverse environments. Likewise, vibrios could increase their resistance to stress via association with biotic surfaces, for instance, by establishing commensal relationships with some zooplankton populations (Colwell & Huq 1994, Pruzzo et al. 2008).

Acknowledgements. This work was supported by the Spanish Ministry of Economy and competitiveness (CGL2015-70929-R), the Basque Government (Spain) (grant BFI-2011-85 to C.P.) and the University of the Basque Country (Spain) (project GIU14/22 and grant PIF15/101 to E.O.), and the Basque Foundation for Science, Ikerbasque (Spain).

LITERATURE CITED

Abia AL, Ubomba-Jaswa E, Momba MN (2016) Competitive survival of *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhimurium* and *Shigella dysenteriae* in riverbed sediments. Microb Ecol 72:881–889

Ammerman JW, Fuhrman JA, Hagström A, Azam F (1984) Bacterioplankton growth in seawater: I. Growth kinetics and cellular characteristics in seawater cultures. Mar Ecol Prog Ser 18:31–39

Armada SP, Farto R, Pérez MJ, Nieto TP (2003) Effect of temperature, salinity and nutrient content on the survival

responses of *Vibrio splendidus* biotype I. Microbiology 149:369–375

Asakura H, Ishiwa A, Arakawa E, Makino S, Okada Y, Yamamoto S, Igimi S (2007) Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state. Environ Microbiol 9:869–879

Atkinson D, Ciotti BJ, Montagnes DJS (2003) Protists decrease in size linearly with temperature: ca. 2.5% °C^{-1}. Proc R Soc B 270:2605–2611

Atlas RM, Bartha R (1998) Microbial ecology. Fundamentals and applications, 4th edn. Benjamin/Cummings Science Publishers, New York, NY

Austin B, Zhang XH (2006) *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett Appl Microbiol 43:119–124

Beardsley C, Pernthaler J, Wosniok W, Amann R (2003) Are readily culturable bacteria in coastal North Sea waters suppressed by selective grazing mortality? Appl Environ Microbiol 69:2624–2630

Beveridge OS, Petchey OL, Humphries S (2010) Mechanisms of temperature-dependent swimming: the importance of physics, physiology and body size in determining protist swimming speed. J Exp Biol 213:4223–4231

Booth IR, Blount P (2012) The MscS and MscL families of mechanosensitive channels act as microbial emergency release valves. J Bacteriol 194:4802–4809

Cardinaud M, Barbou A, Capitaine C, Bidault A, Dujon AM, Morag D, Paillard C (2014) *Vibrio harveyi* adheres to and penetrates tissues of the European abalone *Haliotis tuberculata* within the first hours of contact. Appl Environ Microbiol 80:6328–6333

Chávez-Dozal A, Gorman C, Erken M, Steinberg PD, McDougald D, Nishiguchi MK (2013) Predation response of *Vibrio fischeri* biofilms to bacterivorus protists. Appl Environ Microbiol 79:553–558

Clinical and Laboratory Standard Institute (2017) M100-27. Performance standards for antimicrobial susceptibility testing, 27th edn. Clinical and Laboratory Standards Institute, Wayne, PA

Colwell RR, Huq A (1994) Environmental reservoir of *Vibrio cholerae*. The causative agent of cholera. Ann N Y Acad Sci 740:44–54

Coutard F, Crassous P, Droguet M, Gobin E, Colwell RR, Pommepuy M, Hervio-Heath D (2007) Recovery in culture of viable but nonculturable *Vibrio parahaemolyticus*: Regrowth or resuscitation? ISME J 1:111–120

Davis BJK, Jacobs JM, Davis MF, Schwab KJ, DePaola A, Curriero FC (2017) Environmental determinants of *Vibrio parahaemolyticus* in the Chesapeake Bay. Appl Environ Microbiol 83:e01147-17

Delaney MP (2003) Effects of temperature and turbulence on the predator-prey interactions between a heterotrophic flagellate and a marine bacterium. Microb Ecol 45:218–225

Di DYW, Lee A, Jang J, Han D, Hur HG (2017) Season-specific occurrence of potentially pathogenic *Vibrio* spp. on the southern coast of South Korea. Appl Environ Microbiol 83:e02680-16

Dunn AK, Millikan DS, Adin DM, Bose JL, Stabb EV (2006) New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression *in situ.* Appl Environ Microbiol 72:802–810

Eguchi M, Fujiwara E, Miyamoto N (2000) Survival of *Vibrio anguillarum* in freshwater environments: Adaptation or debilitation? J Infect Chemother 6:126–129

Eiler A, Johansson M, Bertilsson S (2006) Environmental influences on *Vibrio* populations in northern temperate and boreal coastal waters (Baltic and Skagerrak Seas). Appl Environ Microbiol 72:6004–6011

Falcioni T, Papa S, Campana R, Manti A, Battistelli M, Baffone W (2008) State transitions of *Vibrio parahaemolyticus* VBNC cells evaluated by flow cytometry. Cytometry B Clin Cytom 74B:272–281

Faruque SM, Bin Naser I, Islam MJ, Faruque ASG and others (2005) Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. Proc Natl Acad Sci USA 102:1702–1707

Fuhrman JA (1999) Marine viruses and their biogeochemical and ecological effects. Nature 399:541–548

Fukui Y, Saitoh S, Sawabe T (2010) Environmental determinants correlated to *Vibrio harveyi*-mediated death of marine gastropods. Environ Microbiol 12:124–133

Gauthier MJ (2000) Environmental parameters associated with the viable but nonculturable state. In: Grimes DJ, Colwell RR (eds) Nonculturable organisms in the environment. ASM Press, Washington, DC, p 87–112

Girard L, Peuchet S, Servais P, Henry A, Charni-Ben-Tabassi N, Baudart J (2017) Spatiotemporal dynamics of total viable *Vibrio* spp. in a NW Mediterranean coastal area. Microbes Environ 32:210–218

Goikoetxea N, Borja A, Fontán A, González M, Valencia V (2009) Trends and anomalies in sea-surface temperature, observed over the last 60 years, within the southeastern Bay of Biscay. Cont Shelf Res 29:1060–1069

Hahn MW, Höfle MG (2001) Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiol Ecol 35:113–121

Haldar S, Maharajan A, Chatterjee S, Hunter SA and others (2010) Identification of *Vibrio harv*eyi as a causative bacterium for a tail rot disease of sea bream *Sparus aurata* from research hatchery in Malta. Microbiol Res 165: 639–648

Hibbing ME, Fuqua C, Parsek MR, Peterson SB (2010) Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol 8:15–25

Hobbie JE, Daley RJ, Jasper S (1977) Use of nucleopore filters for counting bacteria by epifluorescence microscopy. Appl Environ Microbiol 33:1225–1228

Ishigaki T, Sleigh MA (2001) Grazing characteristics and growth efficiencies at two different temperatures for three nanoflagellates fed with *Vibrio* bacteria at three different concentrations. Microb Ecol 41:264–271

Jacquet S, Miki T, Noble R, Peduzzi P, Wilhelm S (2010) Viruses in aquatic ecosystems: important advancements of the last 20 years and prospects for the future in the field of microbial oceanography and limnology. Adv Oceanol Limnol 1:97–141

Johnson CN (2013) Fitness factors in vibrios: a mini-review. Microb Ecol 65:826–851

Kaberdin VR, Montánchez I, Parada C, Orruño M, Arana I, Barcina I (2015) Unveiling the metabolic pathways associated with the adaptive reduction of cell size during *Vibrio harveyi* persistence in seawater microcosms. Microb Ecol 70:689–700

Kaspar CW, Tamplin ML (1993) Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl Environ Microbiol 59:2425–2429

Kirchhoff C, Cypionka H (2017) Propidium ion enters viable cells with high membrane potential during live-dead staining. J Microbiol Methods 142:79–82

Levitus S, Antonov JI, Boyer TP, Stephens C (2000) Warming of the world ocean. Science 287:2225–2229

Lutz C, Erken M, Noorian P, Sun S, McDougald D (2013) Environmental reservoirs and mechanisms of persistence of *Vibrio cholera.* Front Microbiol 4:375

Massana R, Gasol JM, Bjørnsen PK, Blackburn N and others (1997) Measurement of bacterial size via image analysis of epifluorescence preparations: description of an inexpensive system and solutions to some of the most common problems. Sci Mar 61:397–407

Matz C, Kjelleberg S (2005) Off the hook - how bacteria survive protozoan grazing. Trends Microbiol 13:302–307

Montánchez I, Arana I, Parada C, Garaizabal I, Orruño M, Barcina I, Kaberdin VR (2014) Reprogramming of *Vibrio harveyi* gene expression during adaptation in cold seawater. FEMS Microbiol Ecol 87:193–203

Nowakowska J, Oliver JD (2013) Resistance to environmental stresses by cells of *Vibrio vulnificus* in the VBNC state. FEMS Microbiol Ecol 84:213–222

Oliver JD (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 34:415–425

Oliver JD (2015) The biology of *Vibrio vulnificus.* Microbiol Spectr 3:VE-0001-2014

Oliver JD (2016) The viable but nonculturable state for bacteria: status update. Microbe 11:159–164

Parada C, Orruño M, Kaberdin V, Bravo Z, Barcina I, Arana I (2016) Changes in the *Vibrio harveyi* cell envelope subproteome during permanence in cold seawater. Microb Ecol 72:549–558

Pomeroy LR, Wiebe WJ (2001) Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. Aquat Microb Ecol 23:187–204

Pruzzo C, Vezzulli L, Colwell RR (2008) Global impact of *Vibrio cholerae* interactions with chitin. Environ Microbiol 10:1400–1410

Ramamurthy T, Ghosh A, Pazhani GP, Shinoda S (2014) Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. Front Public Health 2:103

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379

Rowe I, Elahi M, Huq A, Sukharev S (2013) The mechano-electrical response of the cytoplasmic membrane of *Vibrio cholerae.* J Gen Physiol 142:75–85

Sherr EB, Sherr BF (2002) Significance of predation by protists in aquatic microbial food webs. Antonie van Leeuwenhoek 81:293–308

Sherr BF, Sherr EB, Rassoulzadegan F (1988) Rates of digestion of bacteria by marine phagotrophic protozoa: temperature dependence. Appl Environ Microbiol 54: 1091–1095

Sime-Ngando T (2014) Environmental bacteriophages: viruses of microbes in aquatic ecosystems. Front Microbiol 5:355

Soto W, Lostroh CP, Nishiguchi MK (2010) Physiological responses to stress in the *Vibrionaceae.* In: Seckbach J, Grube M (eds) Symbioses and stress. Cellular origin, life in extreme habitats and astrobiology, Vol 17. Springer, Dordrecht, p 407–426

Sun F, Chen J, Zhong L, Zhang XH, Wang R, Guo Q, Dong Y (2008) Characterization and virulence retention of viable but nonculturable *Vibrio harveyi.* FEMS Microbiol Ecol 64:37–44

Sun S, Kjelleberg S, McDougald D (2013) Relative contributions of *Vibrio* polysaccharide and quorum sensing to the resistance of *Vibrio cholerae* to predation by heterotrophic protists. PLOS ONE 8:e56338

Sun S, Tay QXM, Kjelleberg S, Rice SA, McDougald D (2015) Quorum sensing-regulated chitin metabolism provides grazing resistance to *Vibrio cholerae* biofilms. ISME J 9:1812–1820

Takemura AF, Chien DM, Polz MF (2014) Associations and dynamics of *Vibrionaceae* in the environment, from the genus to the population level. Front Microbiol 5:38

Urakawa H, Rivera ING (2006) Aquatic environment. In: Thompson FL, Austin B, Swings J (eds) The biology of vibrios. ASM Press, Washington, DC, p 175–189

Vezzulli L, Colwell RR, Pruzzo C (2013) Ocean warming and spread of pathogenic vibrios in the aquatic environment. Microb Ecol 65:817–825

Wommack KE, Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. Microbiol Mol Biol Rev 64:69–114

Wong HC, Wang P (2004) Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. J Appl Microbiol 96: 359–366

Xu HS, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR (1982) Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb Ecol 8:313–323

Yang Y, Xiang Y, Xu M (2015) From red to green: the propidium iodide-permeable membrane of *Shewanella decolorationis* S12 is repairable. Sci Rep 5:18583

Editorial responsibility: Josep Gasol, Barcelona, Spain

Submitted: October 11, 2018; Accepted: May 6, 2019
Proofs received from author(s): July 1, 2019