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Cell phenotype changes and oxidative stress response in *Vibrio* spp. induced into viable but non-culturable (VBNC) state

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Abstract

Purpose Aquatic bacteria of the genus *Vibrio* include animal and human pathogens. The occurrence of *Vibrio*-related diseases has been associated with the current climate change-driven increase of sea surface temperature. *Vibrio* spp. can enter into the viable but non-culturable (VBNC) state, as a consequence of starvation in seawater at low temperatures. In such physiological state, *Vibrio* cells are no longer culturable on standard media agar plates but can resuscitate if incubated at 30 °C prior to plating, retaining virulence. Since limited information is available on regards to this topic, in this work, we characterized the phenotypic changes of four *Vibrio* spp. strains (one laboratory strain and three environmental isolates) in cold seawater microcosms, investigating the relationship between resuscitation and a hydrogen peroxide-induced oxidative stress.

Methods Cell phenotypic changes and the effect of hydrogen peroxide and/or catalase addition to the medium were studied on VBNC and resuscitated cells by flow cytometry in microcosm experiments, paralleled by culturability experiments by plating.

Results The cells of all the *Vibrio* strains changed their phenotype upon the induction of the VBNC state resulting in cell dwarfing and decrease in DNA quantity, losing the ability to grow on solid media. These features were partially or totally reverted when the cells were treated for resuscitation. Hydrogen peroxide at concentrations as low as 0.007 mM prevented resuscitation and a prolonged exposure to hydrogen peroxide at concentrations far under those inhibiting the growth of log-phase cells permanently damaged VBNC cells, which could not be resuscitated. However, the potential of culturability of VBNC cells could be preserved, at least for a part of the population, by plating the cells in the presence of catalase. The study also showed that during the resuscitation process, the cells gradually increased their resistance to hydrogen peroxide.

Conclusions The timing and mode of induction of the VBNC state, as well as cell resuscitation and response to hydrogen peroxide, differed among *Vibrio* strains, indicating that induction and resuscitation from dormancy could vary in the context of species belonging to a single genus.

Keywords Dormancy, Resuscitation, Culturability, Viability, Hydrogen peroxide, Catalase, Flow cytometry, Oxidative stress

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Introduction

Under unfavourable environmental conditions, microbial cells may enter in a physiological reversible state of low metabolic activity known as quiescence or dormancy (Lennon and Jones 2011). This represents an adaptive strategy for long-term survival of bacteria facing unfavourable environmental conditions (Ayrapetyan et al. 2015). Examples of cellular processes leading to dormancy are sporulation in gram-positive bacteria and the formation of persister cells or viable but non-culturable (VBNC) cells in gram negatives (Lennon and Jones 2011). Specifically, VBNC state is a dormant physiological condition of resistance against stress factors, in which bacterial cells are alive but no longer able to grow on laboratory media; they however retain the capability to restart growth upon "resuscitation" under certain favourable environmental conditions (Lennon and Jones 2011; Oliver 2005). Stress conditions, such as starvation and incubation at nonoptimal temperatures, can induce the VBNC state in a wide range of bacteria, including human pathogens (Oliver 2005, 2010). VBNC cells resist to multiple antimicrobial treatments (e.g. antibiotics) and easily bypass microbiological safety controls due to their lack of culturability, representing thus a potential threat for human health (Nowakowska and Oliver 2013; Li et al. 2014). For instance, wastewater disinfection treatments can induce the VBNC state (Cai et al. 2021). The reuse of such treated wastewater in agriculture and the lack of detection of VBNC cells, which is largely based on cultivation approach, represent a risk not only for the spread of pathogens but also of the antibiotic resistance genes (ARGs) the dormant cells may harbour (Cai et al. 2021; Riva et al. 2020). Furthermore, we have to consider that a number of bacteria for open-field environmental applications are in most cases poorly characterized in relation to their potential VBNC state that may be induced under harsh conditions they may experience during their application in the field (Prakash et al. 2013). Thus, improving the knowledge on the mechanisms leading to or removing the VBNC state is necessary for both human and animal health and to improve the biotechnological exploitation of microorganisms.

VBNC state has been extensively studied in the gram-negative, aquatic bacteria of the genus *Vibrio* (Oliver 2005), which include waterborne or foodborne pathogens (e.g. *Vibrio vulnificus*, *V. cholerae*, *Vibrio haemolyticus*). Such bacteria inhabit marine, freshwater and estuarine environments and can be associated with shellfish and other aquatic animals (O'Neil et al. 1992; Lutz et al. 2013). The current warming of the sea surfaces has been connected with the spread of this genus and the emergence of related human diseases (Vezzulli et al. 2016).

As mentioned above, several stress conditions, such as starvation and incubation at nonoptimal temperatures, can induce the VBNC state. Within *Vibrio* genus, the appearance of VBNC cells has been intensively studied in the species *V. vulnificus*, shortly after incubation in cold seawater; cell resuscitation from this state has been discovered upon exposure to higher temperatures prior to plating (Oliver 2005). The loss of culturability induced by starvation and low temperatures has been associated with significant decrease in the expression of the periplasmic catalase KatG and of catalase activity, which affect the cell resistance to oxidative stress (Kong et al. 2004). Therefore, the growth of VBNC cells in rich media that contain a certain amount of hydrogen peroxide, e.g. brain heart infusion (BHI), is hampered (Kong et al. 2004). On the other hand, the quorum-sensing molecule AI-2, as well as cell-free supernatants from *V. vulnificus*, *Vibrio parahaemolyticus* or *Escherichia coli*, can trigger the resuscitation process in both seawater samples and experimental VBNC microcosms (Ayrapetyan et al. 2014). It has been proposed that increased amounts of AI-2 stimulate the expression of the stress-related alternate sigma factor *rpoS* (through the action of LuxR), restoring the expression of catalase (*katG*) and the culturable state (Ayrapetyan et al. 2014). However, studies in other *Vibrio* species suggest that the mechanisms behind the loss of culturability could be far more complicated (Li et al. 2014). In *V. cholerae*, the induction of VBNC state and the following resuscitation cannot always be obtained as for *V. vulnificus*, but depending on the time spent under the VBNC state, other factors should be provided, such as catalase or human colon cells extracts, or the cells should be co-cultured with colon cells, to rescue from the VBNC state (Imamura et al. 2015). A current view postulates that in *V. cholerae*, the VBNC state is a condition that continuously changes over time, rather than a single-gene property (Imamura et al. 2015). This view is supported by the occurrence of transcriptional changes in the first 12 h of incubation of *V. harveyi* in seawater without nutrients (Montánchez et al. 2014), with cells (which are still culturable) becoming smaller and adapting their gene expression (Kaberdin et al. 2015). The reduction in size and the decrease of DNA quantity have been extensively described also in VBNC cells of *V. parahaemolyticus* (Falcioni et al. 2008; Wagley et al. 2021).

The oxidative stress response has been repeatedly associated with the VBNC state (Li et al. 2014), though the role of hydrogen peroxide is still under debate. On one hand, VBNC cells of clinical strains of *V. vulnificus* have been reported to be highly sensitive to hydrogen peroxide, proposed as the main reason for the inability to grow on BHI (Kong et al. 2004; Nowakowska and Oliver

2013). On the other hand, VBNC cells of an environmental strain of *V. vulnificus* have been shown to be more resistant to this stress than the log-phase cells (Nowakowska and Oliver 2013). During the initial stages of starvation, *V. harveyi* upregulates the genes responsible for the oxidative stress resistance, detoxifying the endogenous hydrogen peroxide generated as a side product of the lipid turnover and repairing the damaged molecules (Kaberdin et al. 2015).

Considering cell response in relation to the oxidative stress caused by hydrogen peroxide, limited information is available about changes occurring in VBNC and resuscitated cells. The aim of this study is thus to assess the relationship between cell phenotypic changes and response to oxidative stress of different *Vibrio* strains during the transition to and from the VBNC state. To this aim, a first part of the work was dedicated to characterize the phenotypic changes of 4 *Vibrio* strains (namely, the laboratory strain *Vibrio campbellii* BAA-1117™ and the 3 environmental strains of *Vibrio hepatarius* UU21, *Vibrio japonicus* UU24 and *Vibrio japonicus* M5) during the incubation in cold seawater microcosms under starving conditions. In the second part of the work, 2 out of 4 strains (i.e. *Vibrio campbellii* BAA-1117™ and *Vibrio japonicus* UU24) were further monitored until the cells completely entered in VBNC state, and their resuscitation was thus induced by incubation at 30 °C for different times before plating. These two strains were selected because the former was the quickest to enter into the VBNC state, while the latter was the one with a distinguishable VBNC kinetic among the other isolates. In these strains, the relationship between resuscitation and the oxidative stress caused by hydrogen peroxide was also investigated.

Materials and methods

Isolation and molecular identification of *Vibrio* spp. strains

One laboratory strain and three environmental isolates of *Vibrio* spp. were used in the study. The laboratory strain was *Vibrio campbellii* BAA-1117™ (luxN::tn5Kan), with a mutation in the LuxN receptor, which is involved in the quorum sensing mediated by homoserine lactones. Strain BAA-1117™ is a derived mutant of strain BAA-1116™ that was reclassified as *Vibrio campbellii* according to microarray comparative genome hybridization and multilocus sequence analysis (Lin et al. 2010). Three environmental strains, UU21, UU24 and M5, were isolated from the gills of the fiddler crab *Tubuca urvillei*, sampled in 2011 in Gazi Bay mangrove forest (−4.416, 39.51; Kenya). For bacterial isolation, the crab gills were dissected in proximity of a Bunsen burner flame, placed in a 2-ml tube where the gills were washed in sterile physiological solution (9 g/L NaCl) to remove external particles. After preliminary washing, the gills were smashed in sterile saline (9 g/L NaCl), and serial dilutions were plated on Marine Agar (Difco) plates added with 100 µg/ml cycloheximide to avoid eukaryotic growth. Plates were incubated aerobically at 30° C for 24–48 h. Once the growth occurred, random colonies were selected and purified. The 16S rRNA gene of the isolates was amplified through PCR using primers 27F (5′-TCGACATCG TTTACGGCGTG-3′) and 1492R (5′-CTACGGCTACCT TGTTACGA-3′) (Mapelli et al. 2013) and sequenced at Macrogen (South Korea). The obtained sequences were aligned against the curated database of EZBioCloud (https://www.ezbiocloud.net/; Yoon et al. 2017) to confirm their affiliation to *Vibrio* spp. Sequences were then deposited in the ENA database under the accession number ERZ12298639. Glycerol stocks were stored at −80 °C.

Preparation of *Vibrio* microcosms and induction of the VBNC state

Strains were grown overnight at 30 °C in tryptic soy broth added with 25 g/L NaCl (TSB-NaCl). Bacterial cultures were used to inoculate (1% v/v) fresh TSB-NaCl broth. As soon as the cultures reached the exponential growth phase (optical density, OD, 0.3–0.5), cells were washed twice in sterile artificial sea water (ASW). To prepare ASW, 40 g of sea salts (Sigma-Aldrich, Milan, Italy) was dissolved in 1 L of deionized water, and the solution was filtered with 0.22 µm pore size filters (Millipore, Milan, Italy). Cells were counted using a Thoma chamber and diluted in sterile ASW to reach the concentration of approximately 1×10^7 cells/ml in glass vials. Microcosms prepared in ASW and thus, in starvation condition, were stored at 4 °C in the dark; starvation and low temperature were applied to induce the VBNC state. To check the entrance in VBNC state by cultivation, culturability trials were carried out by drop-plate technique. Briefly, culturable cells collected from microcosms, initially shaken to allow the aggregate breakage and sample homogenization, were counted in 10 µl of decimally diluted triplicated aliquots plated on TSB-NaCl plates (added with 15 g/L agar), incubated overnight at 30 °C. Viability staining was assessed by flow cytometry, as reported below.

Assessment of VBNC state and resuscitation

In order to verify that the observed increase in culturable cells was due to resuscitation and not to the growth of an originally undetectable non-VBNC cell population, we applied the procedure adapted by Whitesides and Oliver (1997). An aliquot of the microcosm was shaken and serially diluted, and a sample of each of the dilutions, as well as of the undiluted sample, were plated (100 µl) on TSB-NaCl agar plates to verify the absence of growth. We incubated the remaining dilutions overnight at 30 °C to

induce resuscitation, since temperature upshift has been described to resuscitate VBNC cells, e.g. in *V. vulnifucus* (Oliver 2005). The following day, we plated (100 µl) each dilution on TSB-NaCl agar plates, and, after a further overnight at 30 °C, the number of colonies was assessed. Assuming that, if the increase in CFU counts was due to the growth of originally undetectable non-VBNC cells rather than to the resuscitation, at least one non-VBNC cell should have been initially present in each dilution from which we cultivated cells, we then calculated the theoretical initial concentration of these non-VBNC cells in the undiluted sample and verified that this value was higher than our detection result (Whitesides and Oliver 1997).

For other experiments of resuscitation from the VBNC state, we incubated the bacterial aliquots at 30 °C for different time spans prior to plating on TSB-NaCl agar plates and let them grow overnight.

Viability staining and flow cytometry

Two aliquots of 100 µl were collected from each microcosm and stained separately with SYTO™ 9 or propidium iodide (PI) from the LIVE-DEAD BacLight kit (L7012, Life Technologies, Milan, Italy). Both live, and dead bacterial cell membranes are permeable to SYTO™ 9, which binds DNA and RNA. PI only enters dead or damaged cells. The staining solutions were prepared by adding 3 µl of each dye to 1 ml Milli-Q water, according to the manufacturer protocol; then, 100 µl of the staining solutions was mixed with each microcosm aliquot. Samples were analysed in flow cytometry using a BD Accuri™ C6 device (BD Biosciences, Milan, Italy). For each sample, 30,000 events were acquired, and a non-stained sample from each microcosm was always included in the analysis. The excitation laser was set at 488 nm. Green fluorescence signal of SYTO™ 9 was detected with a 530/30 nm filter, while red fluorescence from PI was measured with a 585/40 nm filter. In our experimental setup, the use of PI to trace dead cells based on red fluorescence was not useful; we could conversely detect a green fluorescent stain using PI (see "Results" section). The obtained data were analysed using BD Accuri™ C6 Plus software version 1.0 (BD Biosciences, Milan, Italy). Flow cytometry analyses were used to evaluate phenotypic characteristics of *Vibrio* strains in VBNC state and after resuscitation.

Plates treated with catalase and determination of strains' catalase production

Catalase from bovine liver (C9322) was purchased from Sigma-Aldrich (Milan, Italy), dissolved in phosphate buffer pH 7 at the concentration of 1 mg/ml and filter sterilized. In order to obtain the same catalase activity per plate as described by Kong et al. (2004), 1 ml of catalase solution was poured on each TSB-NaCl agar plate. The plates were dried in a biosafety cabinet and immediately inoculated with 100 µl of VBNC cells. The number of colonies was evaluated after an overnight incubation at 30 °C.

The production of catalase by the four *Vibrio* strains was visually assessed using the straightforward method described by Iwase et al. (2013). Briefly, a calibration curve was constructed with defined units of catalase in a solution made of 1% (v/v) Triton X-100 and undiluted hydrogen peroxide (30% v/v); the height of oxygen-forming foam was measured using a ruler. Samples of bacterial cells (10 mg), prepared using overnight cultures, were resuspended in physiological saline and subjected to the same procedure (Iwase et al. 2013).

Growth in the presence of hydrogen peroxide of VBNC or resuscitated cells

To determine the sensitivity of the strains to hydrogen peroxide, growth curves of standard-growing cells of the strains BAA-1117™ and UU24 were obtained using the Infinite® 200 PRO microplate reader (TECAN Italia S.r.l, Milan, Italy). With the term "standard-growing cells/cultures", we identified cells of bacterial strains routinely grown in the lab (i.e. not induced in VBNC state or resuscitated from VBNC state). Ten microlitres of the cell suspension (10^6 cell/ ml) was inoculated in 90 µl TSB-NaCl medium in each well of a 384-well microplate using an ep-Motion liquid handler (Eppendorf, Milan, Italy). Different concentrations of hydrogen peroxide (from 0 to 0.6 mM) were prepared by adding to each well 2 µl of hydrogen peroxide solution at the appropriate concentrations diluting 30% (v/v) hydrogen peroxide (Sigma-Aldrich, Milan, Italy) in deionized water. The microtiter plates were incubated at 30 °C for 24 h, and the optical density was measured every 15 mins. We calculated the growth curves by averaging the OD measurements of 8 replicates and subtracting the average OD value of negative controls (non-inoculated wells). We performed an end-point analysis counting the number of wells showing the bacterial growth at the end of the incubation time.

The capability of cells from microcosms to grow in the presence of hydrogen peroxide was monitored in microtiter plates using the protocol reported above. Specifically, we considered (i) cells collected from microcosms at day 3, before the induction of the VBNC state (inoculum: 10 µl; hydrogen peroxide concentrations from 0 to 0.600 mM), (ii) VBNC cells (inoculum: 10 µl; hydrogen peroxide concentrations from 0 to 0.250 mM) and (iii) resuscitated VBNC cells (inoculum: 10 µl; hydrogen peroxide concentrations from 0 to 0.250 mM), following the incubation at 30 °C for different resuscitation times.

Resuscitation after stress hydrogen peroxide

Two-millilitre aliquots of the microcosms were prepared in sterile tubes. To each one, the appropriate volume of hydrogen peroxide/water solution was added to reach the final hydrogen peroxide concentration of 0.007 mM, 0.02 mM and 0.05 mM. After overnight incubation at 30 °C, an aliquot of cell suspension was plated, while a second one was treated with catalase (C9322 Sigma-Aldrich; adding 25 µl of catalase solution to 1 ml aliquot: stock 1 mg/ml) and incubated at 30 °C until plating the following day. Cell phenotypic characteristics were analysed by flow cytometry, as reported above.

Results

Starvation in cold seawater induces phenotypic changes

Vibrio strains isolated from the mangrove crab gills were identified as *Vibrio hepatarius* UU21, *Vibrio japonicus* UU24 and *Vibrio japonicus* M5 by partial 16S rRNA gene sequencing and alignment against the curated database EZBioCloud (Table 1; Fig. S1). To monitor phenotypic changes in the cells subjected to starvation and cold stresses, we analysed, by flow cytometry, the cells from microcosms at different times during a time span of 50 days, upon staining with the LIVE-DEAD BacLight kit; at each analysed time point, we plated the cells to also assess their culturability.

For all the strains, we observed the same trends, although with a different timing. In all the microcosms, a cell shrinkage as decreasing of the FSC-A parameter was observed by flow cytometry (Fig. 1A–D), while in case of *V. campbellii* BAA-1117™, this process was already completed at day 33, and for strains *V. hepatarius* UU21 and *V. japonicus* UU24 and M5, we observed a dwarf population at day 47/50, even if large cells were still present (Fig. 1A–D; Fig. S2). Similarly, the green fluorescence signal detected upon staining with SYTO™ 9, which is proportional to DNA abundance into the cells, decreased rapidly in *V. campbellii* BAA-1117™ and more slowly in the other strains (Fig. 1E–H).

Concomitantly to the reduction in size and DNA quantity, we recorded an upshift of the green fluorescence upon staining with propidium iodide (PI) in the dwarf cells or the smallest fraction of cells (in size) (Fig. 1I–L; Fig. S2). Experiments with cells artificially killed by heat shock (10 min at 100 °C) or by adding 50% (v/v) isopropanol demonstrated that in our experimental setup, PI was unable to confer a red fluorescence to the dead cells in the microcosms. It is noteworthy to remark that *Vibrio* cells, grown in routine laboratory conditions and killed by heat or isopropanol, showed red fluorescence with PI staining, highlighting that the lack of red fluorescence for the PI-treated killed cells was specific for cells collected from microcosms. Moreover, no green fluorescence signals were detected in PI-treated killed cells, demonstrating that the PI-green fluorescence was related to live cells.

In *V. campbellii* BAA-1117™, all the cells showed increased value of green fluorescence upon staining with PI already at day 33, while for the other strains, two populations appeared: one with lower size cells and higher green fluorescence and the other with bigger cells but less fluorescent upon staining with PI (Fig. 1I–L; Fig. S2).

Microcosms of *V. campbellii* BAA-1117™ and *V. japonicus* UU24 were monitored until they completely entered into VBNC state (Fig. 1). Strain BAA-1117™ completely lost the capability to generate colonies on TSB-NaCl plates at day 53 while strain UU24 at day 108 (Figs. 1M and 2N). VBNC-induced cells of the 2 strains BAA-1117™ and UU24 showed, by flow cytometry, cell populations with smaller size, lower DNA content and higher green fluorescence upon staining with PI at days 50 and 124 of incubation, respectively (Fig. 1; Fig. S2).

Table 1 Identification of the isolates obtained in the study

Isolate	EZBioCloud type strain
UU24	*Vibrio japonicus* JCM 31412
UU21	*Vibrio hepatarius* LMG 20362
M5	*Vibrio japonicus* JCM 31412

Resuscitation completely reverted the cell phenotype for strain BAA-1117™

To resuscitate the cells, we applied the protocol described by Whitesides and Oliver (1997) incubating an aliquot of the microcosm at 30 °C overnight; plating the cell suspension after this treatment resulted in colony growth on TSB-NaCl agar medium. In order to demonstrate that the observed colonies appeared after resuscitation rather than being related to the growth of a previously undetectable fraction of non-VBNC-induced cells (i.e. to verify that all the cells included into microcosms were in VBNC state), we serially diluted the VBNC-induced microcosm prior to incubation at 30 °C and still in starvation conditions, and we verified that cells resuscitated in all the dilutions. Dilutions were indeed plated immediately to verify the absence and presence of CFUs, respectively, before and following the resuscitation process at 30 °C. Resuscitation occurred in all dilutions incubated at 30 °C, and in the case of *V. campbellii* BAA-1117™, the final colony counts reached levels which reflected the dilution applied. For instance, for this strain after 48 h of resuscitation, we counted ~9×10^4, 9×10^3 and 9×10^2 cells/ml in the dilutions 1:100, 1:1000 and 1:10,000, respectively (Whitesides and Oliver 1997). Assuming that they can be grown from a single non-VBNC parental cell,

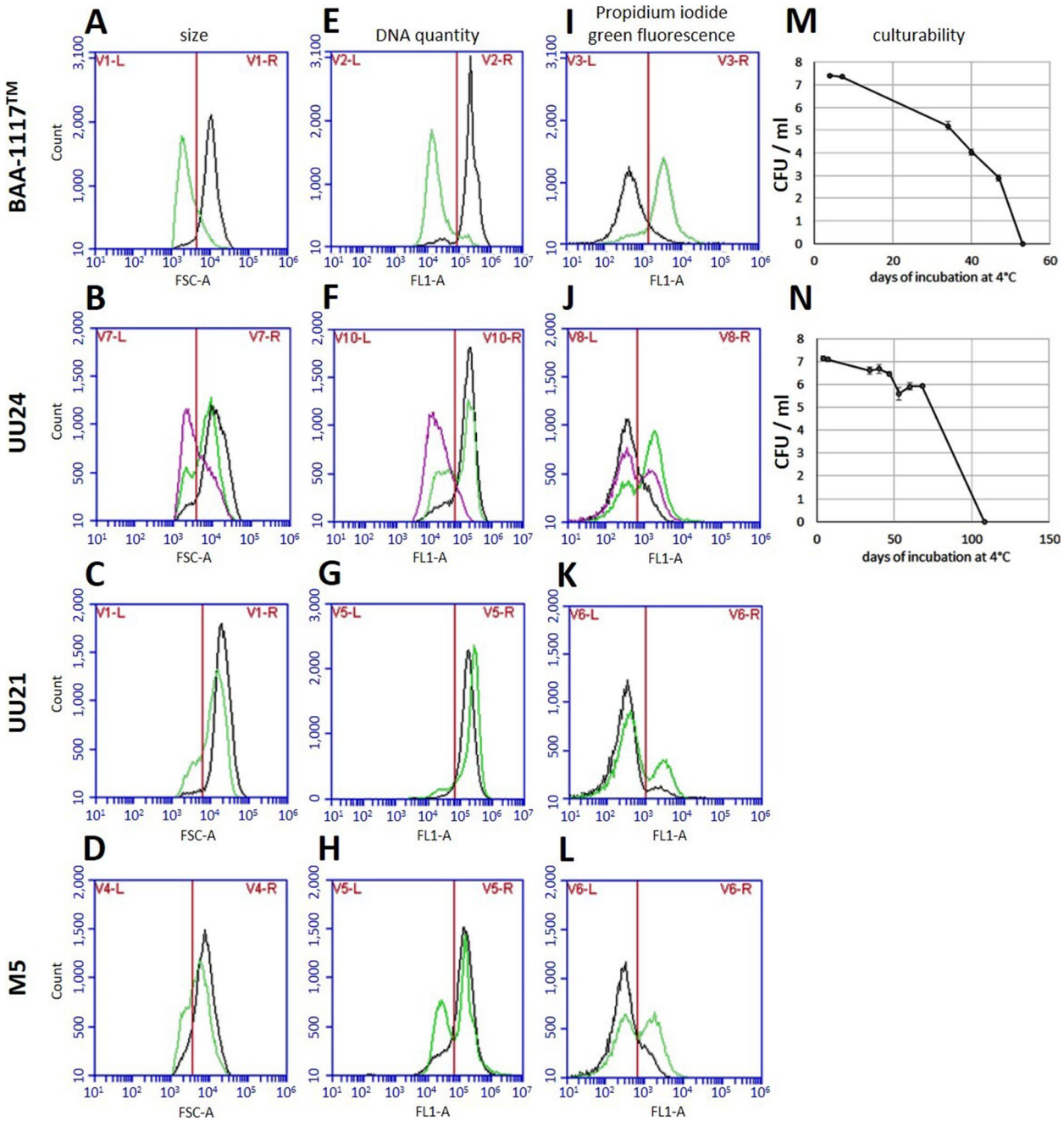

Fig. 1 Change in size, fluorescence and culturability of *Vibrio* spp. strains entering in VBNC state. Changes detected by flow cytometry. **A**–**D** Cell size (FSC-A). **E**–**H** DNA quantity (green fluorescence (FL1-A) of cells stained with SYTO™ 9). **I**–**L** Green fluorescence (FL1-A) of cells stained with propidium iodide (PI). Over time, the cell size decreases, as well as the DNA quantity, which is proportional to the green fluorescence of SYTO™ 9; concomitantly, the green fluorescence induced by PI increases. **M**–**N** Decrease in culturability during incubation at 4 °C, according to the CFU numbers detected by plating cells on solid medium. CFU counts are reported as Log_{10}(CFU + 1). **A**, **E**, **I** and **M** *V. campbellii* BAA-1117™ strain. **B**, **F**, **J** and **N** *V. japonicus* UU24 strain. **C**, **G** and **K** *V. hepatarius* UU21 strain. **D**, **H** and **L** *V. japonicus* M5 strain. Black line: microcosm at 3 days. Green line: microcosm at 50 days. Purple line: microcosm at 124 days

there should have been in the original microcosm at least 9×10^6 culturable cells/ml, which were not detected in the plating before the treatment. We observed a similar trend of resuscitation in the case of *V. japonicus* UU24: we were still able to count the resuscitated cells in the more diluted samples, but we noticed a confluent growth

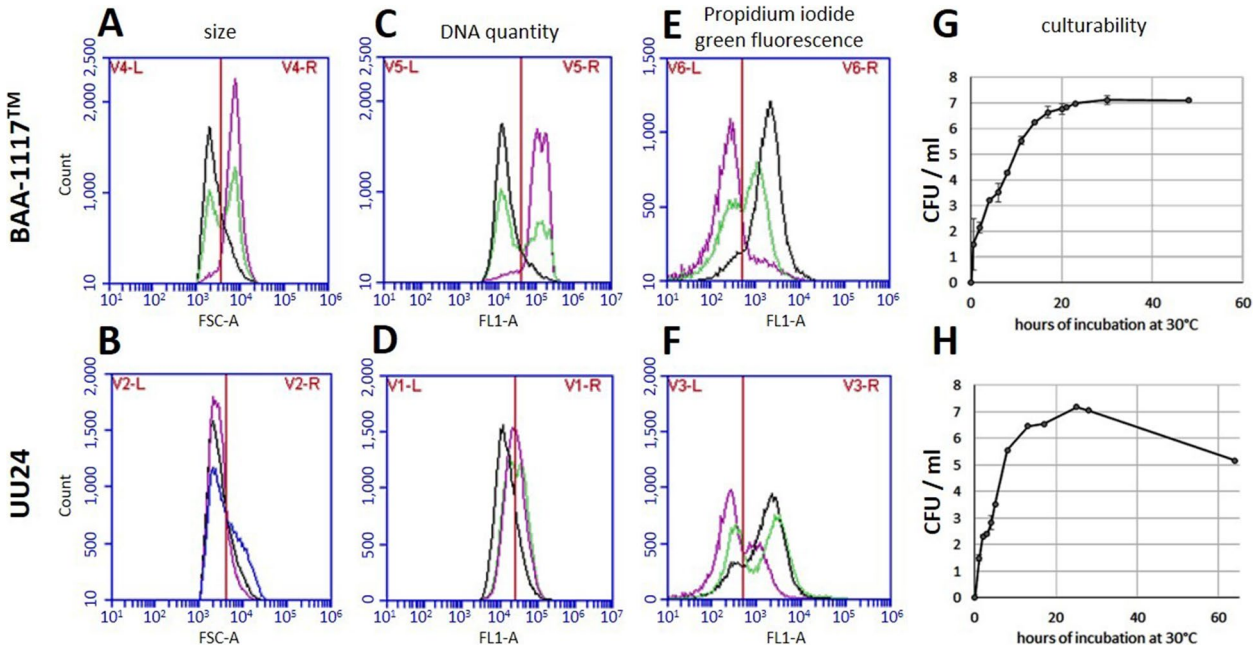

Fig. 2 Change in size, fluorescence and culturability of *Vibrio* spp. strains exiting from VBNC state. Resuscitation observed by flow cytometry. **A**–**B** Cell size (FSC-A). **C**–**D** DNA quantity (green fluorescence (FL1-A) of cells stained with SYTO™ 9). **E**–**F** Green fluorescence (FL1-A) of cells stained with propidium iodide (PI). Changes detected before for cell size, DNA quantity and propidium iodide fluorescence (Fig. 1) are reverted in a different extent in the two strains. Resuscitation detected by culturability. **G**–**H** Increase in culturability, according to the CFU numbers detected plating cells on solid medium, after the incubation at 30 °C. The process of resuscitation happens exponentially. Counts (CFU/ml) are reported as Log_{10}(CFU + 1). **A**, **C**, **E** and **G** *V. campbellii* BAA-1117™ strain. Black line: no incubation at 30 °C. Green line: after 17 h of incubation at 30 °C. Purple: after 21 h of incubation at 30 °C. **B**, **D**, **F** and **H** *V. japonicus* UU24 strain. Blue line: no incubation at 30 °C. Black line: after 1 h of incubation at 30 °C. Green line: after 24 h of incubation at 30 °C. Purple line: after 64 h of incubation at 30 °C

of cells by plating those less diluted ones for which it was supposed to find single colonies; no cell colonies were however detected in the plating before the resuscitation treatment. Nevertheless, it remains to be elucidated why strain UU24 showed a confluent growth when those dilutions were resuscitated.

Resuscitation of *V. campbellii* BAA-1117™ and *V. japonicus* UU24 was monitored over time. Microcosms' aliquots were incubated at 30 °C along different times, after which the cells were observed by flow cytometry and plated (Fig. 2G–H). In both strains, the CFU increase was correlated to the time of incubation at 30 °C (resuscitation time) with an exponential relationship, until CFU number reached a plateau. However, flow cytometry revealed a different behaviour of the two strains. In *V. campbellii* BAA-1117™, the decrease in size and DNA quantity and the increase in PI-green fluorescence resulted completely to revert after resuscitation with the process completed between 17 and 21 h (Fig. S3). After 17 h, two populations were still detectable: one smaller in size and with an increased fluorescence upon staining with PI and one larger in size (resuscitated) which showed a decrease of fluorescence (Fig. 2 A, C, E; Fig. S3). Concomitantly, the green fluorescence upon staining with SYTO™ 9 increased (Fig. 2C). The same reversal was observed in *V. japonicus* UU24, but was not completed in the monitored time (Fig. 2B, D, F; Fig. S3); the PI-green fluorescence gradually decreased (Fig. 2F), but cells were still distributed in two populations of different fluorescence after 64 h (Fig. S3). DNA quantity and cell size did not increase significantly (Fig. 2B and D).

Catalase induced the resuscitation of VBNC cells

According to Kong and co-workers (2004), we verified if the treatment of TSB-NaCl plates with catalase could restore the culturability of VBNC cells. This was carried out with aliquots of cells from the microcosms of *V. japonicus* UU24 at 179 days and *V. campbellii* BAA-1117™ at 31 days. Although the latter was not yet completely in VBNC state, the use of catalase greatly improved the culturability of the cells in both cases. However, the number of colonies obtained with this procedure was 100 or 1000 fold lower than the number of colonies obtained by resuscitation at 30 °C (Fig. 3).

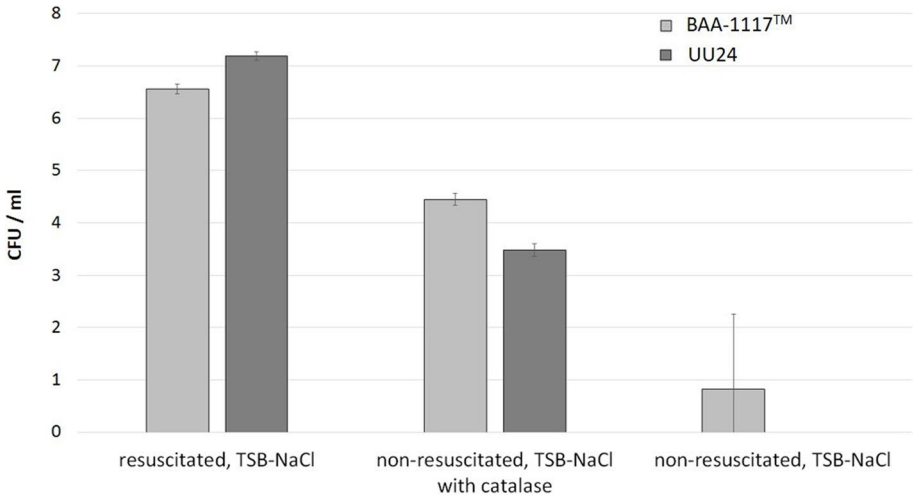

Fig. 3 Culturability of *V. campbellii* BAA-1117™ (light grey) and *V. japonicus* UU24 (dark grey). Different treatments were considered: (i) after resuscitation on TSB-NaCl agar plates, (ii) without resuscitation on TSB-NaCl agar plates treated with catalase and (iii) without resuscitation on TSB-NaCl agar plates. Cells from *V. campbellii* BAA-1117™ microcosm were not yet completely in VBNC state (31 days). Cells from *V. japonicus* UU24 microcosm were in VBNC state (179 days). Use of catalase greatly improved the culturability of the cells for both strains. Significant differences were observed among treatments for each strain (*t*-test, $p < 0.05$). Counts (CFU/ml) are reported as $Log_{10}(CFU + 1)$

VBNC cells are sensitive to hydrogen peroxide

To assess the capability of VBNC cells to grow in the presence of oxidative stress, we first challenged standard-growing cells with different concentrations of hydrogen peroxide (Fig. S4). We noticed that the response to hydrogen peroxide was discrete, which means that only in some wells we could observe bacterial growth. The growth rate could be also influenced by the presence of hydrogen peroxide (i.e. we observed a delay of growth in the presence of certain concentrations of hydrogen peroxide, as shown in Fig. S5). Thus, to assess the strains' response to hydrogen peroxide, we counted the number of wells in which strains' growth was observed. Standard-growing cells of *V. campbellii* BAA-1117™ and *V. japonicus* UU24 showed a different sensitivity to hydrogen peroxide. Strain BAA-1117™ growth failed in 25% of the wells added with 0.1 mM hydrogen peroxide, and it was consistently inhibited with 0.2 mM hydrogen peroxide (Fig. S4). Conversely, strain UU24 showed a higher resistance to oxidative stress induced by hydrogen peroxide: at 0.1 mM hydrogen peroxide, it always grew as good as in the absence of hydrogen peroxide, while 0.225–0.250 mM hydrogen peroxide could prevent the growth of the strain in 25% of the wells (Fig. S4). This behaviour was observed for cells collected from the microcosms at day 3 (i.e. cells not yet entered into VBNC state).

Conversely, when in VBNC state, cells of both strains could not grow in the presence of any of the hydrogen peroxide concentrations applied. Sensitivity to hydrogen peroxide was then checked by exposing the VBNC cells at different periods of resuscitation at 30 °C and in the presence of hydrogen peroxide concentrations from 0.05 to 0.25 mM (Fig. 4). According to our data, the capability to cope with oxidative stress was gradually acquired with the resuscitation time. After 7–16 and 5–20 h of resuscitation, respectively, *V. campbellii* BAA-1117™ and *V. japonicus* UU24 displayed a behaviour similar to non-VBNC cells. After 20 h of resuscitation, *V. campbellii* BAA-1117™ resulted even more resistant to oxidative stress, growing in all the wells containing 0.1 mM hydrogen peroxide (Fig. 4).

Hydrogen peroxide prevents resuscitation by damaging cells that cannot be rescued by catalase in the plate growth medium

To further investigate the relationship between resuscitation and sensitivity to hydrogen peroxide, we added hydrogen peroxide (0.007, 0.02 and 0.05 mM) to microcosms' aliquots prior to the incubation of VBNC cells at 30 °C for resuscitation. According to Kong et al. (2004), a hydrogen peroxide concentration of 0.007 mM, which is present in the standard cultural media, is enough to prevent cell growth. We verified that the 0.007 mM concentration prevented the resuscitation (culturability) of VBNC cells of *V. campbellii* BAA-1117™, while for *V. japonicus*, UU24 concentrations higher than 0.02 mM were necessary. Cells did not show an increase in size nor anyone of the changes described before for the resuscitation process (Fig. 5). To verify if they were still capable to resuscitate after relieving the oxidative stress, we added catalase and plated the bacterial

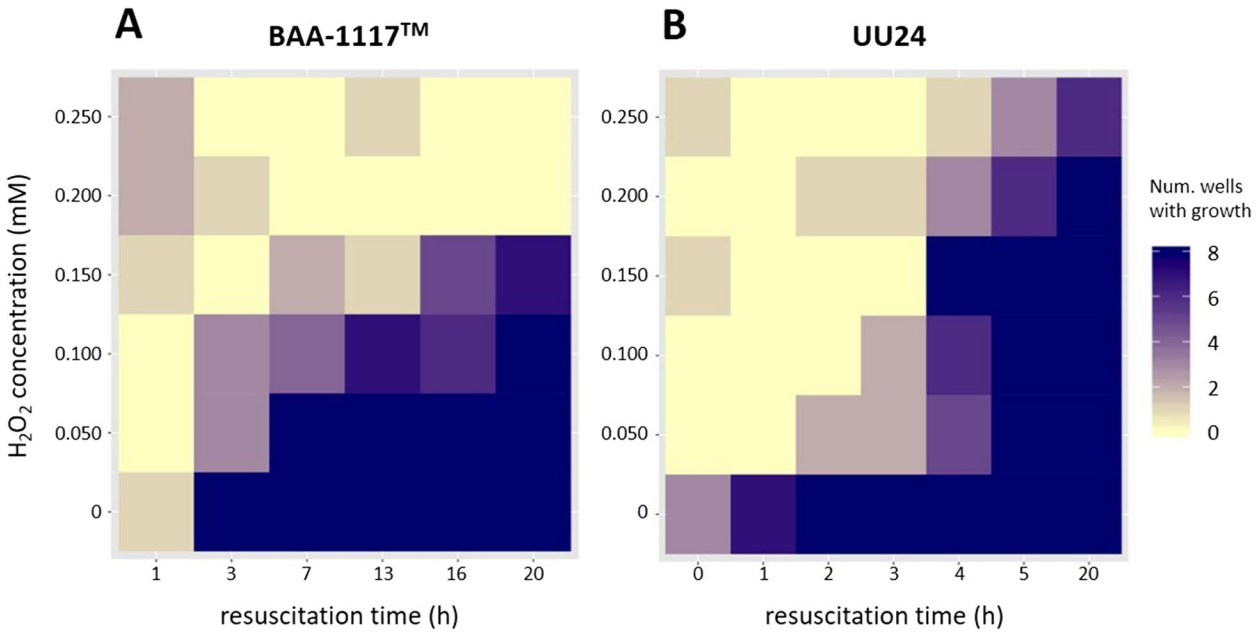

Fig. 4 Heat maps showing the growth of *V. campbellii* BAA-1117™ (**A**) and *V. japonicus* UU24 (**B**). Growth was studied in the presence of increasing hydrogen peroxide concentrations after different resuscitation times (incubation at 30 °C) in a microtiter plate. *X*-axis: hours of incubation at 30 °C; *Y*-axis: H_2O_2 concentrations (mM). Different colours represent number of wells in which growth has been detected based on the increase of OD. Both the strains acquired the capability to overcome oxidative stress gradually during resuscitation, though with a different timing and against different concentrations of hydrogen peroxide

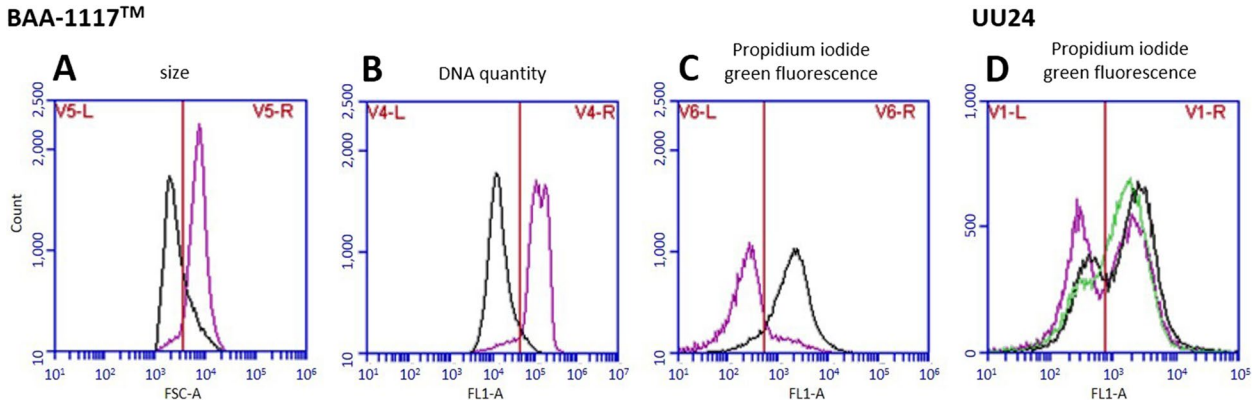

Fig. 5 Size and fluorescence of the VBNC-induced cells of *V. campbellii* BAA-1117™ and *V. japonicus* UU24. Cells were incubated at 30 °C to induce resuscitation in the presence of different hydrogen peroxide concentrations, i.e. oxidative stress. Black line: cells incubated in the presence of 0.007 mM hydrogen peroxide. Green line: cells incubated in the presence of 0.02 mM hydrogen peroxide. Purple line: cells "resuscitated" at 30 °C and inserted to indicate the hypothetical shift in case of resuscitation. If resuscitation had occurred, black and green lines would have been merged with purples ones. **A**, **B** and **C** *V. campbellii* BAA-1117™. While the resuscitated cells increased in cell size and DNA quantity and decreased in green fluorescence when stained with PI (please check Fig. 1), stressed cells preserved the phenotypic features of VBNC cells. **A** Size (FSC-A). The size of stressed cells did not increase as that of resuscitated cells. **B** Green fluorescence (FL1-A) of cells stained with SYTO™ 9. The quantity of DNA in stressed cells did not increase. **C** Green fluorescence of cells stained with propidium iodide. The fluorescence of stressed cells did not decrease. **D** *V. japonicus* UU24; green fluorescence of cells stained with propidium iodide. While in resuscitated cells a big population has lost the fluorescence, in stressed cells, this population is much smaller. While cells stressed with 0.007 mM hydrogen peroxide were still able to resuscitate and grow, cells stressed with higher concentrations of hydrogen peroxide are not. Size and DNA quantity did not show in this strain any appreciable difference between resuscitated, VBNC and stressed cells

suspensions; cells could not resuscitate after a further overnight incubation in presence of catalase, probably because they were permanently damaged by the prolonged exposure to the oxidant.

Catalase production

Only *V. campbellii* BAA-1117™ showed high catalase production (207 units for 10 mg cells) with the selected method (Iwase et al. 2013), while for the other three environmental strains (UU21, UU24 and M5), catalase production appeared negligible if compared to the one of *V. campbellii* BAA-1117™.

Discussion

The time to enter a VBNC state is highly variable in the genus *Vibrio*, 3 (Kong et al. 2004) to 4 days (Whitesides and Oliver 1997) for *V. vulnificus* and 60 (Asakura et al. 2007) to 77 days (Imamura et al. 2015; Senoh et al. 2015) for *V. cholerae*. *V. harveyi* cells were followed for 21 days, but the induction of VBNC state was not reported as complete (Parada et al. 2016; Kaberdin et al. 2015; Montánchez et al. 2014). Variable times are reported also for *V. parahaemolyticus* from 9 (González-Escalona et al. 2006) to 69 days (Falcioni et al. 2008). Such differences may be attributed to the specificity of the strains or of the experimental conditions. Our experiments, performed on three *Vibrio* strains isolated from environmental samples and one collection strain (*V. campbellii* BAA-1117™), confirmed that the time to enter a VBNC state is variable for the different species tested and can be a very slow process, involving for some strains only a fraction of the cells. All *V. campbellii* BAA-1117™ cells rapidly and almost simultaneously lost their culturability and acquired specific features when observed by flow cytometry (Fig. 1). Consistent with the observations reported in Falcioni et al. (2008), the size of VBNC cells markedly decreased, as well as DNA content (Trevors et al. 2010). At the same time, when stained with PI, VBNC cells acquired an unexpected green fluorescence, which in our experiments appeared to be consistent with VBNC state induction (as discussed later). In the other three strains (UU24, M5 and UU21), we observed the same behaviour, even though with a remarkably slower rate (Fig. 1).

PI is a well-known red-fluorescent cationic dye used to detect cells with damaged membranes. Although it is not clear if cells could actually recover after the damage (Davey and Hexley, 2011), it is commonly used to detect dead cells, often as part of live/dead kits (Falcioni et al. 2008; González-Escalona et al. 2006; Imamura et al. 2015). However, under the experimental conditions adopted in this study, we did not detect any red fluorescence upon staining with PI over time in ASW microcosms. Thus, we were not able to assess the number of damaged cells. However, observing the fluorescence of PI-stained cells in the green channel (FL1), we found that the dwarf, low-DNA population that appeared as a consequence of prolonged incubation in cold ASW recorded an increase in green fluorescence: this could be possibly linked to some changes in the cells exposed to starvation, which affect the fluorophore labelling (Parada et al. 2016; Kaberdin et al. 2015; Montánchez et al. 2014). The green fluorescence appeared only in the aliquots stained with PI, but not in the non-stained ones, and such observation was consistently repeated for all the strains. Moreover, during the resuscitation process, the green fluorescence was related to cells exiting from VBNC state, while in *V. campbellii* BAA-1117™, the decrease in fluorescence has been combined with other changes; in *V. japonicus* UU24, this was the only phenotypic feature useful to trace the resuscitation process. Indeed *V. japonicus*-resuscitated cells did not increase their size or DNA quantity but lost the PI-induced green fluorescence. This phenomenon is rather difficult to explain but yet consistent in our data. Further investigations are required to clarify this observation; indeed, the discovery of a marker of VBNC or resuscitated *Vibrio* cells would be of great interest both from public health and marine ecology perspectives.

The well-known "great plate count anomaly" states that only a minor fraction of the bacterial species in the environment is culturable (Connon and Giovannoni 2002). Our findings pointed out that, also in case of culturable strains and dormant cells, plate count could underestimate the bacterial load of an environmental sample. While all the cells of *V. campbellii* BAA-1117™ acquired the features of VBNC state in a short time span (53 days), among the considered strains, for our environmental strains, the VBNC state entrance appeared to be a slower process (e.g. 108 days for strain UU24) than for the laboratory strain and eventually involved only a fraction of the total cell population. However, as reported above, the time to enter a VBNC state is highly variable in *Vibrio* genus and reported to be, for instance, 3–4 days for *V. vulnificus* and 60–77 days for *V. cholerae* (Kong et al. 2004; Whitesides and Oliver 1997; Asakura et al. 2007; Imamura et al. 2015; Senoh et al. 2015). Thus, even if we count a number of colonies of a certain species from an environmental sample, we cannot exclude that other populations are present in the sample, and other approaches should be paralleled to cultivation for confirmation.

When VBNC cells of *V. campbellii* BAA-1117™ were resuscitated, the exact reversion of the phenotypic features detected in the process of VBNC state induction was observed (Baffone et al. 2006; Li et al. 2014). For this strain, which was the quickest to enter into VBNC state in our experimental conditions, the resuscitation was also a rapid process involving almost all the cells at

the same time. The resuscitation of *V. japonicus* UU24 was instead slower and gradual (Fig. 2). Although they became again culturable, cells only partially reverted the VBNC state phenotype, particularly considering cell size and DNA quantity. As observed for the entrance into VBNC state, resuscitation of our environmental strains involved only one subpopulation. This confirms previous evidence about the fact that the mechanisms of the resuscitation process vary across strains (Li et al. 2014). This implies that every attempt to improve the culturability of environmental bacteria based on a resuscitation protocol should be aware of the variability among strains. Timing and protocols applied to restore the culturability could affect each strain differently; attempts of resuscitating simultaneously a mixed community, for example from an environmental sample prior to a cultivation-based survey, could hence result in different proportions of resuscitated cells from each of the strains in the sample.

VBNC cells of *V. campbellii* and *V. japonicus* can be also regrown by supplementing the plates with catalase to remove the hydrogen peroxide that is naturally present in the medium (Arana et al. 1992), even if with a lower efficiency than by incubation at 30 °C. Therefore, the oxidative stress induced by the presence of hydrogen peroxide is not the only factor implied in the loss of culturability of these cells, in particular in the case of *V. japonicus* (Li et al. 2014). To clarify the strains' behaviour in the presence of hydrogen peroxide, we measured the sensitivity to this compound of both strains during growth in standard conditions and their ability to produce catalase. Interestingly, although more sensitive to hydrogen peroxide, *V. campbellii* BAA-1117™ showed a catalase production far higher than the environmental strains analysed in this study, suggesting that they could have different mechanisms of protection from oxidative stress. However, after resuscitation, the oxidative stress resistance is gradually acquired along with the time of incubation at 30 °C (Fig. 4).

Kong and colleagues (2004) measured that hydrogen peroxide concentration in plates of Heart Infusion Agar was 0.007 mM. We verified that the addition of this hydrogen peroxide concentration to the microcosms' aliquots, prior to incubation at 30 °C, prevented the resuscitation and growth of *V. campbellii*, which was more sensitive to hydrogen peroxide, but not of *V. japonicus*, which required concentrations approximately three times higher. Nevertheless, in both cases, the concentration that could prevent the resuscitation was far lower than the one that could inhibit growth of standard cultures, confirming the hypothesis that VBNC cells are more sensitive to oxidative stress (Nowakowska and Oliver 2013). This effect was sharper in *V. campbellii*, which possibly relied more on catalase for the protection against oxidative stress. VBNC cells incubated at 30 °C with the addition of hydrogen peroxide did not show any phenotypic change as reported in case of resuscitated cells (Fig. 5). Thus, to assess if they retained the capability to resuscitate or they were dead, we added catalase, and we incubated them at 30 °C for an additional overnight. After plating, we did not observe any colony, suggesting that the exposure to hydrogen peroxide killed the VBNC cells before they could resuscitate.

Conclusions

Data highlighted that hydrogen peroxide is an important factor preventing the growth of VBNC cells in plates, although it was not sufficient to explain the differential response of the tested strains. Its detrimental effects varied in magnitude for the two considered strains of *V. campbellii* BAA-1117™ and *V. japonicus* UU24, which both resulted more sensitive as VBNC cells rather than as growing cells. In this regard, future studies could be focused on the metabolic pathways involved in the resuscitation process of bacteria, when exposed to the oxidative stress imposed by hydrogen peroxide, considering different times of resuscitation and monitoring the bacterial gene expression and proteome profiles. Moreover, it will be important to understand the mechanism explaining why for some species only a subset of clonal populations enter the VBNC state.

In this study, we also observed that *Vibrio* cells incubated in microcosms acquired, over time, a green fluorescence when stained with PI, which then reverted with resuscitation. Interestingly, we found that for *V. japonicus* UU24, the decrease in PI-green fluorescence was the only phenotypic feature useful to trace the resuscitation process. Considering that this behaviour is rather difficult to explain, but consistent in our data, we should remark that the discovery of a marker of VBNC *Vibrio* cells would be of great interest, and that further investigations are needed to clarify this observation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-022-01703-6.

Additional file 1: Figure S1. Multi-alignment analysis of the partial 16S rRNA gene sequences of the 4 *Vibrio* strains used in this study. The multi-alignment was performed with Clustal Omega (EMBL-EBI; https://www.ebi.ac.uk/Tools/msa/clustalo/; version 1.2.4). **Figure S2.** Appearance of dwarf populations for the 4 *Vibrio* spp. strains. Along with a prolonged incubation in cold ASW, a dwarf population appeared, characterized by an increased green fluorescence upon staining with PI. A, B: *V. campbellii* BAA-1117™. C, D: *V. hepatarius* UU21. E, F: *V. japonicus* M5. G, H, I: *V. japonicus* UU24. Plots show the size (x axis) and the PI-green fluorescence (y axis) of the microcosm cells after 3 days of incubation (A, C, E, G) or after 47 days (B, D, F, H). While all *V. campbellii* BAA-1117™ cells are dwarf and fluorescent with PI after 47 days, for the other strains two populations are detectable. For *V. japonicus* UU24, the dwarfing process is completed

in 124 days (I). **Figure S3.** Size (x axis) and green fluorescence (y axis) upon staining with propidium iodide of *V. campbellii* BAA-1117™ and *V. japonicus* UU24 during the resuscitation process. A, B, C: VBNC-induced cells of strain BAA-1117™ after 0, 17 and 21 hours of incubation at 30°C, respectively. D, E, F: VBNC-induced cells of strain UU24 after 1, 24 and 64 hours of incubation at 30°C, respectively. During incubation at 30°C in "resuscitation" process, a less fluorescent population appeared, which in strain BAA-1117™ resulted bigger in size. **Figure S4.** Sensitivity of strains *V. campbellii* BAA-1117™ (A) and *V. japonicus* UU24 (B) to hydrogen peroxide (0 mM – 0.6 mM), visualized by counting the microtiter wells in which the bacterial growth was observed. Overnight cultures of cells (not induced to enter into VBNC state) were used. **Figure S5.** Growth curves of strains *V. campbellii* BAA-1117™ (A) and *V. japonicus* UU24 (B) in presence of increasing quantities of hydrogen peroxide (0 mM – 0.6 mM). The graphs indicate the growth curves generated by the mean O.D. value detected in 8 independent microtiter wells (replicates) for each hydrogen peroxide concentration considered.

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Authors' contributions
EMP and EC conceived the study. EMP, SA, FM, ZZ, MF and EC performed the experiments and analysed the data. MF sampled the crabs. EMP wrote the first version of the manuscript. SB and DD supported the research. All authors critically revised the manuscript and have approved its content.

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Availability of data and materials
Data related to DNA sequencing are made available through public databases as specified in the "Materials and methods" section.

Declarations

Ethics approval and consent to participate
Decapod crustaceans (such as lobsters and crabs) are exempt from current European legislation that protects animals used for scientific purposes because they are non-sentient and thus incapable of suffering even though the care and use of the experimental animals complied with local animal welfare laws, guidelines and policies.

Consent for publication
All of the authors consent to the publication of this manuscript in ***Annals of Microbiology***.

Competing interests
The authors declare that they have no competing interests.

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