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Research Paper

Rapid Detection and Fast Induction of Viable but Nonculturable *Vibrio parahaemolyticus* and *Vibrio cholerae*

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ABSTRACT

Vibrio (*V.*) species, such as *V. parahaemolyticus* and *V. cholerae*, are commonly associated with foodborne infections and are frequently detected in seafood worldwide. Unfavorable environmental conditions and process-related factors can induce a shift from culturable *Vibrio* cells into viable but nonculturable (VBNC) cells.

Conventional culture-based detection methods (ISO 21872-1:2023-06) cannot detect bacteria in the VBNC state, even though these cells remain metabolically active and pathogenic due to the expression of toxin – encoding genes. This study aimed to develop a detection method using viable quantitative PCR (vqPCR) to identify viable cells, including those in VBNC state. In parallel, a relatively rapid protocol for inducing the VBNC state to generate VBNC cell controls was established.

The established vqPCR assays included a preliminary step to inhibit dead bacterial cells using a proprietary DNA intercalating dye (Reagent D) in combination with the detection of long gene fragments of *gro*EL (510 bp) for *V. parahaemolyticus* and *omp*W (588 bp) for *V. cholerae* using previously published primers. These assays demonstrated a high sensitivity, detecting as low as 20 fg DNA = 3.5 *V. parahaemolyticus* cells and 30 fg DNA = 6.9 *V. cholerae* cells. An induction of *Vibrio* VBNC cells of ≈ 6.5 Log10 cells/ml was successfully achieved within one hour from an initial 7.3 Log10 viable *Vibrio* cells/ml by treating the cells with a solution containing 0.5 or 1.0% Lutensol A03 and 0.2 M ammonium carbonate.

The results showed that the established vqPCR methods were able to detect *V. parahaemolyticus* and *V. cholerae* in up to 50% (2.6 to 4.2 Log10 cells/g) and 56% (2.8 to 5.2 Log10 cells/g) of retail samples, respectively, that were initially false-negative in culture-based tests.

The use of vqPCR assays along with culture-based tests can significantly enhance the seafood safety assessment by enabling the detection of VBNC cells of the most important foodborne *Vibrio* pathogens. In addition, the induction assay can be used for a rapid production of VBNC cells to standardize and validate such detection methods.

V. parahaemolyticus and non-O1/non-O139 *V. cholerae* are often associated with human infections traced back to the consumption of contaminated seafood. Especially, the consumption of contaminated undercooked or raw seafood like oysters and sushi is a common cause that results in *Vibrio* infections. This is due to the worldwide prevalence of the Genus *Vibrio* in marine and estuarine ecosystems, where seafood is exposed to these bacteria (Bonnin-Jusserand et al., 2019).

V parahaemolyticus is the leading cause of seafood-associated diarrhea (Daniels et al., 2000). Self-limiting gastroenteritis as well as sporadic outbreaks around the world are associated with this pathogen (Velazquez-Roman et al., 2014; Baker-Austin et al., 2018). V. cholerae serotypes O1 and O139, the causative agents of Cholera, as well as non-O1 and non-O139 V. cholerae likewise cause sporadic cases of gastroenteritis, and localized foodborne outbreaks worldwide (Bagchi et al., 1993; Bhattacharya et al., 1993; Onifade et al., 2011;

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Marin et al., 2013; Arteaga et al., 2020; Ke et al., 2022). In general, the number of infections caused by Vibrio spp. steadily increased worldwide in recent years. This is due to the increasing consumption of fish and seafood, the intensified use of aquaculture, growing global trade, and rising water temperatures (Baker-Austin et al., 2018; Vezzulli et al., 2020; FAO, 2020; FAO, 2022).

Thus, *V. parahaemolyticus* and non-O1/non-O139 *V. cholerae* are regularly detected in seafood worldwide (Odeyemi, 2016; Ma et al., 2023). As a standard diagnostic method, food samples are qualitatively analyzed for *Vibrio* spp. using culture-based detection methods according to DIN/EN/ISO 21872-1:2023-06. Culture-independent molecular biological detection methods are still based on a prior culture-dependent enrichment as well as obtaining pure cultures. However, the viable but nonculturable (VBNC) state, which is usually triggered by exogenous stress, poses a problem in the culture-based *Vibrio* diagnostic tests. Unfavorable environmental and process-related conditions such as low temperatures or the lack of nutrients can lead to a shift from culturable *Vibrio* cells to cells in VBNC state. As mentioned, bacteria in the VBNC state are not detectable in food samples via culture-based methods, although they are still metabolically active and remain pathogenic due to the expression of toxin−encoding genes such as cholera toxin gene (*ctx*) and hemolysin genes (*tdh, trh*) (Baffone et al., 2003; Oliver, 2005; Asakura et al., 2007; Oliver, 2010; Mishra, Taneja & Sharma, 2012). Thus, *V. parahaemolyticus* and *V. cholerae* in VBNC state pose a public health risk in e.g. chilled and frozen seafood products (Liao, Zhao & Wang, 2017).

Consequently, culture-independent detection methods are needed to detect VBNC *V. cholerae* and *V. parahaemolyticus* cells from seafood products (Fleischmann et al., 2021). Previous studies already established VBNC cell detection assays using viable quantitative real-time PCR (vqPCR) approaches using DNA intercalating dyes for *V. parahaemolyticus* (Zhu et al., 2012; Zhang et al., 2015; Niu et al., 2018; Liu et al., 2018; Yoon et al., 2019; Di Salvo et al., 2023) and *V. cholerae* (Wu, Liang & Kan, 2015). However, standardization and validation of methods without the inclusion of cells in VBNC state is challenging because false-positive or negative results are difficult to evaluate. Previously established vqPCR assays have been based either on the differentiation between viable and dead bacterial cells or on the use of VBNC cells induced via cold-starvation, a process that can take several days to months to induce the VBNC state. Robben et al. (2018) offer a rapid method to induce the VBNC state in *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* Typhimurium, and *Staphylococcus aureus* within one hour only by treating cells in combined solutions of non-ionic surfactants and inorganic salts. To the best of our knowledge, such a rapid method for inducing the VBNC state in *Vibrio* spp. has not yet been reported. This advancement opens new possibilities for employing such cells as standardized controls in the development, validation, and optimization of VBNC detection methods. In vqPCR, VBNC cells can be detected in contrast to dead cells, as the pretreatment with DNA-intercalating dyes selectively penetrates cells with compromised membranes, binding to their DNA and preventing its amplification, while leaving the DNA of viable VBNC cells with an intact cell membrane unaffected.

This study therefore pursues two main objectives. First, to establish a quantitative detection method of only viable *V. parahaemolyticus* and *V. cholerae* cells, including VBNC cells directly from seafood samples to ensure the safety of these products. Second, to develop a rapid VBNC induction assay for *V. parahaemolyticus* and *V. cholerae*, analogous to the method described by Robben et al. (2018) to enable the generation of VBNC cells which can be used as real VBNC controls.

Material and methods

Bacterial cultivation and DNA extraction for standard curves. *V parahaemolyticus* strain RIMD 2210633 and *V. cholerae* non-O1/non-O139 strain ATCC 14730 were used as positive controls as well as for generating qPCR standard curves. These strains were stored at −80 °C in cryotubes (Mast Diagnostica) and cultured on Lysogeny Broth agar (LB agar, Merck) at 37 °C for 24 h. Overnight cultures were prepared using individual colonies from LB agar plates, which were transferred to liquid LB medium (Merck) and incubated for 18 h at 37 °C. Afterwards, 1 ml was taken for DNA extraction using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instruction. The quality and quantity (ng/µL) of DNA were determined by spectroscopy (NanoDrop 2000c, Thermo Scientific) to prepare a defined tenfold serial dilution for the standard curves. It was ensured that the initial concentration was always the same, even if a new DNA extraction from pure culture was used, to generate reproducible standard curves. Extracted DNA was stored at 4 °C until use.

Singleplex qPCR for detection of *V. parahaemolyticus* and *V. cholerae*. To validate and standardize the singleplex qPCR for detecting *V. parahaemolyticus* and *V. cholerae,* standard curves were generated using serial dilutions of extracted DNA from *V. parahaemolyticus* and *V. cholerae* control strains. The standard curve demonstrated a linear relationship between threshold cycle (Ct) values and Log10 DNA starting quantity (ng/µL). The species-specific gene targets of highly conserved genes were amplified in seven independent experiments and during each sample examination. Each reaction was conducted in technical triplicates. Species-specific, sensitive, and accurate gene targets used, identifying exclusively *V. parahaemolyticus* or *V. cholerae,* were originally published by Nandi et al. (2000) and Hossain et al. (2012) based on conventional PCR protocols. These conventional PCR assays were adapted into quantitative PCR assays, and the qPCR reaction mix was prepared as follows: One qPCR reaction had a total volume of 12.5 µl comprising a mixture of final concentrations of 2x SSoFast™ EvaGreen Mastermix, 0.6 pM of each primer and 3 µl DNA (<50 ng). PCR conditions were 95 °C for 180 s followed by 40 amplification cycles including a denaturation for 30 s at 95 °C and annealing/elongation for 60 s for *omp*W at 60 °C or for *gro*EL at 68 °C. The subsequent melting curve (5 s at 65 to 95 °C) was used to identify specific melt peaks at 86 °C for *gro*EL and 82.5 °C for *omp*W. Only a single peak at the expected melting temperature is indicative of a specific qPCR product, as demonstrated by Nandi et al. (2000) and Hossain et al. (2012) by the detection of a single band at the expected bp size in an agarose gel. The long PCR products of 510 bp of the target gene *gro*EL for *V. parahaemolyticus* as described by Nandi et al. (2000) and 588 bp of the target gene *omp*W for *V. cholerae* as described by Hossain et al. (2012) were selected to ensure selective detection of target genes from cells with intact cell membrane and to suppress blocked DNA during live/dead discrimination (see section 2.3). The qPCR was performed using the CFX96 optical reaction module with the C1000 thermal cycler (Bio-Rad) and the CFX manager 3.0 (Bio-Rad) software. In addition to a specific product, the accuracy and sensitivity of the qPCR assay were examined as well. Correlation coefficients (R^2), slopes (s) of standard curves, and the amplification efficiencies (E) were calculated as described by Rasmussen (2001). The R^2 value should be >0.99, an optimal slope of the standard curve between −3.1 and −3.6, and E ≤ 95%. Accordingly, the sensitivity of qPCR was verified from the tenfold serial dilution of each target gene according to Caraguel et al. (2011). The Ct value distribution within technical replicates (n = 3) of each dilution should have a standard deviation (SD) of <0.5. For biological replicates (n = 7), a SD of Ct value distribution should be ≤1.0.

Combined live/dead differentiation with subsequent DNA extraction. Live/dead differentiation of bacterial cells were prepared using Reagent D (Hygiena) according to the manufacturer's instructions. Reagent D binds covalently on freely accessible DNA and DNA from membrane-compromised dead cells to inhibit DNA amplification and avoid false-positive PCR results. Therefore, this assay enables the selective quantification of viable cells with an intact cell membrane including cells in VBNC state. All samples were divided into two

groups: untreated and Reagent D – treated samples. For the inhibition of unbound Reagent-D, the foodproof D-Light LED lamp (Hygiena) was used. Afterwards, bacterial DNA (with and without Reagent D treatment) was extracted using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. All DNA preparations were stored at 4 °C until use.

Induction and conformation of cells in VBNC state. In order to induce the VBNC state of viable *V. parahaemolyticus* and *V. cholerae* cells, the method by Robben et al. (2018) was adapted. Robben et al. (2018) reported a method to induce the VBNC state in *L. monocytogenes*, *E. coli*, *S.* Typhimurium, *Staph. aureus,* and toxin-producing enteropathogenic *E. coli* by treating the bacterial cells with a combined solution of nonionic surfactants, commonly used as cleaners in food-processing environments, and inorganic salts. In this study, 17 nonionic surfactants in combination with three different inorganic salts were tested to induce the VBNC state in *V. parahaemolyticus* and *V. cholerae* cells (see section 3.2).

An initial OD_{600} of 0.6 of a bacterial culture, which resulted in 7.3 log10 viable cells/ml in vqPCR, was used to generate cells in the VBNC state (Fig. 1). Each treatment mixture had a total volume of 1 ml and contained final concentrations of either 1 or 0.5% nonionic surfactant, either 1 or 0.5 M $MgCl_2$, or 0.2 M $(NH_4)_2CO_3$ or 0.1 M K_2CO_3, and bacterial culture at an OD_{600} of 0.06 corresponding to 6.3 log10 viable cells.

The induction of the VBNC state was determined by the culturability of the bacterial cells on LB, the metabolic activity using the API 20E Kit (BioMérieux) and the BacTiter-Glo Microbial Cell Viability Assay (Promega), and the membrane integrity using the vqPCR assays established in this study and the LIVE/DEAD *BacLight* bacterial viability kit (Molecular Probes) directly after the treatment by washing bacterial cells in LB and after 24 h incubation in LB at 37 °C. All kits were used according to the manufacturer's instructions and as described by Robben et al. (2018).

Preparation of food samples. To identify VBNC *Vibrio* spp. in naturally contaminated food samples, 74 cooled and frozen seafood samples (n = 65 shrimp and n = 9 mussel samples) from retail markets in Germany were analyzed (n = 38 for *V. parahaemolyticus* and n = 36 for *V. cholerae*). Samples were transferred to the laboratory under cold conditions and analyzed within 24 h. In addition to qualitative detection of *V. parahaemolyticus* and *V. cholerae* via enrichment culture methods according to DIN/EN/ISO 21872-1:2023-06, samples were quantitatively analyzed by cultivation on thiosulfate citrate bile sucrose agar (TCBS, Oxoid) and culture – independent detection using vqPCR to discriminate VBNC from culturable cells. One hundred grams of each sample was homogenized and divided into aliquots of 25 g. Each aliquot was diluted 1 to 10 with alkaline saline peptone water (ASPW, Merck) for enrichment culture and homogenized (SmacherAE-SAP1064, BioMerieux) for 1 min at the lowest level. For vqPCR, 1 ml was transferred from the homogenized sample in a separate tube. Live/dead differentiation and DNA extraction were followed as described in section 2.3.

Calculations, evaluation, and statistical analysis. For accuracy and reproducibility within real-time amplification, Ct values were directly used and geometric and arithmetic means as well as standard deviation (SD) were calculated. Linear range was defined between the highest and the lowest quantifiable Log10 DNA starting quantity (ng/µl) of standard templates and threshold cycle (Ct) values established by means of a calibration curve. All samples analyzed in this study were present within the linear portion of the calibration curve. The linearity was determined by means of correlation coefficients (R^2). Regression analyses were performed ($y_i = \beta \times x_i + \alpha$), and the slopes (s) were calculated. Reaction efficiency (E) was calculated with the formula $E = (10^{(1/slope)} - 1) \times 100$. A mean standard curve was generated by calculating the cycle thresholds (Ct) and standard deviations (SD) for each individual dilution based on seven independent experiments, and each reaction was conducted in triplicates. This procedure was also performed for each individual vqPCR run, with three technical replicates per standard curve dilution.

The genome size and molar mass of DNA were used for the calculation of bacterial cells e.g. genome equivalents. The genome size of *V. parahaemolyticus* RIMD 2210633 is 5,165,770 bp (Makino et al., 2003). The genome size of non-O1/non-O139 *V. cholerae* is approximately 3,918,007 bp (Leong, Gordon & Rogers, 2018). To calculate the amount of bacterial cells/genome equivalents of 1 ng DNA, the following formula was used: 1 ng DNA = Avogadro constant/(number of nucleotides × weight of nucleotides in a double strand). The molar mass of the DNA was calculated from the mean value of the bp and assumed to be 661.9 g/mol.

To validate Reagent D for vqPCR, 50 µl *V. cholerae* and *V. parahaemolyticus* cells from overnight cultures, dissolved in 350 µl LB, were treated with 150 µl Reagent D both before (viable bacterial cells) and after cell lysis at 95 °C for 20 min (dead bacterial cells) and analyzed using vqPCR. Non-Reagent D – treated samples for both conditions were prepared as well. The number of viable cells was subtracted from

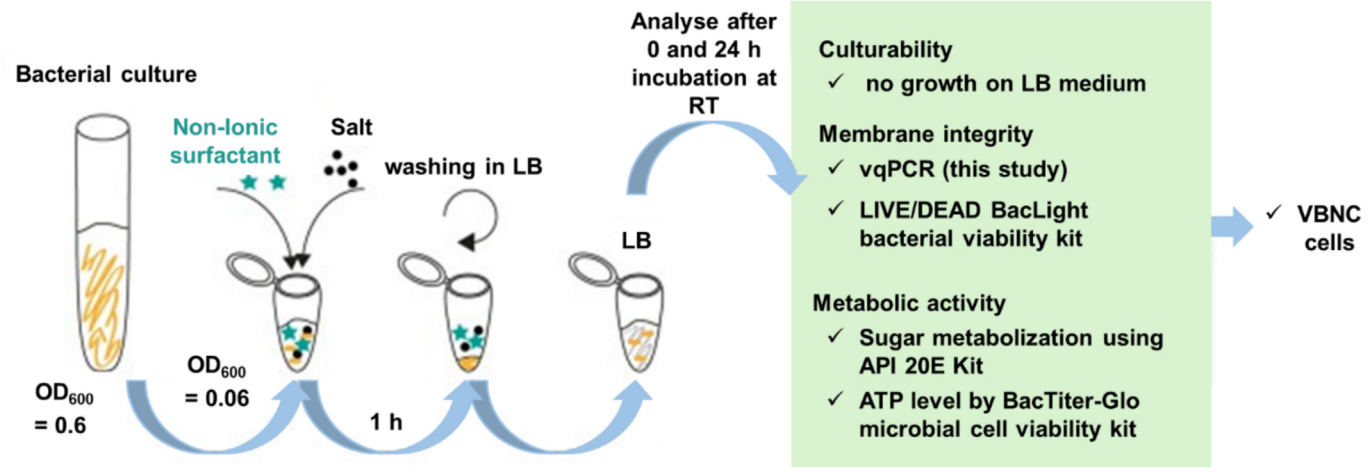

Figure 1. Flow diagram for the induction of VBNC cells according to Robben et al. (2018). A combination of nonionic surfactants and inorganic salts was used to induce the VBNC state of vegetative V. parahaemolyticus and V. cholerae cells. After incubation and washing of cells in LB medium, cells were analyzed using culture-based detection on LB agar and broth, membrane integrity-based assays vqPCR and LIVE/DEAD BacLight viability kit, metabolic activity-based assay API 20E and BacTiter-Glo microbial cell viability kit direct after treatment (0 h) and after 24 h by room temperature (RT) according to the manufacturer's instructions.

the total cell count in the non-Reagent D−treated sample to calculate the number of dead cells in Δlog10.

This resulted in the following scenarios:

(1) Total number of cells: qPCR without Reagent D
(2) Number of viable cells: vqPCR using Reagent D
(3) Number of dead cells: (qPCR without Reagent D) – (vqPCR using Reagent D)

Results

Singleplex vqPCR for detecting *V. parahaemolyticus* and *V. cholerae*. To establish a singleplex vqPCR for the detection of *V. parahaemolyticus* and *V. cholerae*, standard curves were generated using serial tenfold dilution of extracted DNA from *V. parahaemolyticus* strain RIMD 2210633 and *V. cholerae* strain ATCC 14730. The starting DNA quantity (ng/μL) was determined by spectroscopy (NanoDrop 2000c, Thermo Scientific). A strong linear correlation of Ct value and Log10 DNA quantity of *gro*EL and *omp*W target gene standards during amplification was obtained with $R^2 = 0.9948$ and $R^2 = 0.9986$, respectively (Fig. 2). The achieved slope of the standard curve for *gro*EL was −3,41 and for *omp*W −3.35 within the optimal slope range between −3.1 and −3.6. A maximum reaction efficiency of 97% for *gro*EL and 99% for *omp*W with a sensitivity of 20 fg DNA (3.5 bacterial cells) and 30 fg (6.9 bacterial cells), respectively, was achieved. The standard deviation (SD) for each individual dilution within the technical replicates ($n = 3$) was <0.5 for both PCR assays. Within the biological replicates ($n = 7$), the SD of each individual dilution was <0.9 for *V. parahaemolyticus* and <0.8 for *V. cholerae*. Since only a single melting peak was detected in the qPCR and a single band in the agarose gel per reaction, the specificity of both qPCR assays was found to be 1.0.

After establishing the standard curves, the inhibition of membrane-damaged dead bacterial cells and free bacterial DNA was determined to achieve the detection of viable bacterial cells only. For this purpose, a sample volume (1 ml) from an enrichment culture (16 h incubation of a pure culture of *V. parahaemolyticus* and *V. cholerae* at 37 °C in LB medium) was treated with Reagent D according to the manufacturer's instruction for a viable bacterial cell sample, while a second aliquot (1 ml) was boiled at 95 °C for 20 min prior to the Reagent D treatment generating a dead bacterial cell sample. Afterward, the DNA was extracted and the vqPCR assay was performed for both *V. parahaemolyticus* and *V. cholerae*. An example of the results of an analysis for both *Vibrio* species is shown in Figure 3.

In both vqPCR assays, a specific melting peak of the gene targets is available for the samples containing viable bacterial cells (green curves), as DNA from viable cells was amplified and not inhibited by Reagent D. DNA from membrane-damaged bacteria or free DNA in these samples was inhibited by Reagent D, indicating that target gene sequences from this DNA were not amplified. Samples containing only DNA from heat-inactivated bacterial cells (orange curves), which were also treated with Reagent D, showed no specific melting peak of the gene targets, as no target DNA was amplified; these samples can therefore be considered negative. The negative control (blue curves) likewise showed no specific melting peak and can also be considered negative. This indicates that the inhibition of free DNA or DNA of membrane−damaged bacterial cells with Reagent D was successful. Regarding the amplification curves, it is noticeable that both samples with lysed bacterial cells and the negative control rose above the threshold after cycle 35 of the vqPCR. However, a false-positive result can be excluded owing to the melting point analysis. With a shorter vqPCR of 35 cycles, nonspecific amplifications can be avoided.

Induction of VBNC state *V. parahaemolyticus* and *V. cholerae*. In our study, we used the findings of Robben et al. (2018) on other foodborne pathogens to generate VBNC cells of *V. parahaemolyticus* and *V. cholerae* in order to use them as positive controls in the vqPCR assays. In total, 17 nonionic surfactants combined with three different

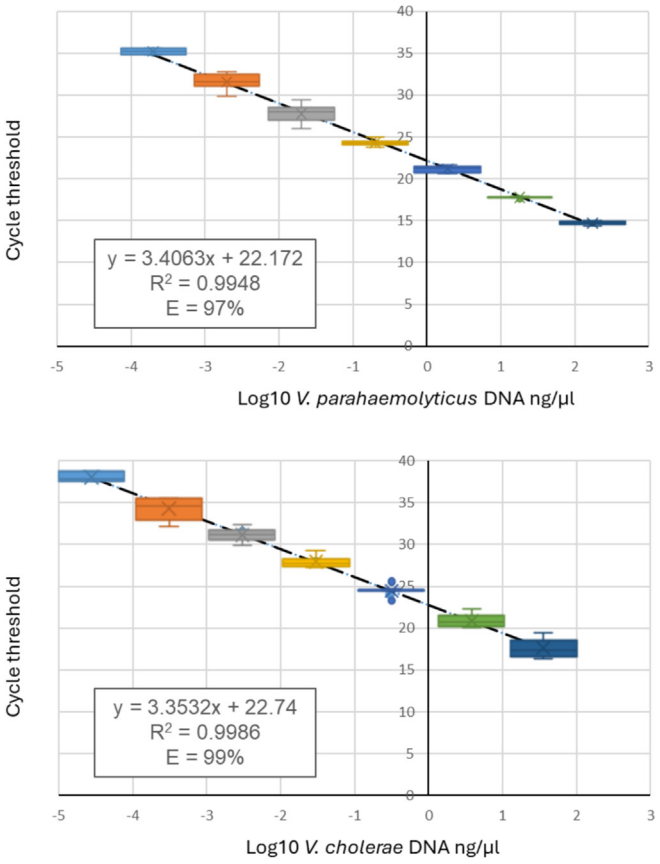

Figure 2. Box-whisker plots represent the range of variation of the Ct values of the individual standards of the target genes *gro*EL for the detection of *V. parahaemolyticus* and *omp*W for the detection of *V. cholerae*. Fluctuations in Ct values of each of the seven individual standards were acquired in seven independent experiments, whereby each reaction was performed in triplicate ($n = 21$ per box-whisker plot). The trend line is shown as a black dashed line. The rectangle shows the slope, the R^2 value, and the efficiency.

inorganic salts were tested (Fig. 1). The VBNC state for *V. parahaemolyticus* and *V. cholerae* cells was directly verified after a resuspension in liquid LB medium for one hour and after 24 h treatment by their culturability, metabolic activity, and membrane integrity (Fig. 1). The VBNC state of *V. parahaemolyticus* cells was successfully induced after an incubation of one hour in 0.5% Lutensol A03 and 0.2 M $(NH_4)_2CO_3$ solved in LB media. For *V. cholerae* a concentration of 1% Lutensol A03 combined with 0.2 M $(NH_4)_2CO_3$ was sufficient. All other combinations of nonionic surfactants and inorganic salts did not induce the VBNC state and led to either culturable cells or cell death (Fig. 4).

The results confirming successful VBNC induction are summarized in Figure 5. The nonculturability of VBNC cells (blue boxes in Fig. 4) on standard culture media was confirmed on LB medium within 24 to 48 h at 37 °C, in contrast to viable cultivable cells (green boxes in Fig. 4), in which growth was observed. In addition to the absence of growth, the metabolic activity of bacterial cells in VBNC state was confirmed via their ability to ferment sugars in the API 20E system and to produce ATP (Fig. 5), as is the case with viable cultivable cells. The fermentation patterns of the VBNC cells showed the same fermentation patterns as the viable culturable cells in the API 20E wells after incubation for 24 h at 37 °C even if they were nonculturable in liquid or on solid LB medium. The VBNC cells were also able to produce ATP after a resuspension for one hour and 24 h in liquid LB medium, as indicated by a luminescence signal above 10,000 relative light units (RLUs), confirming their viability while bacterial cell suspensions below this

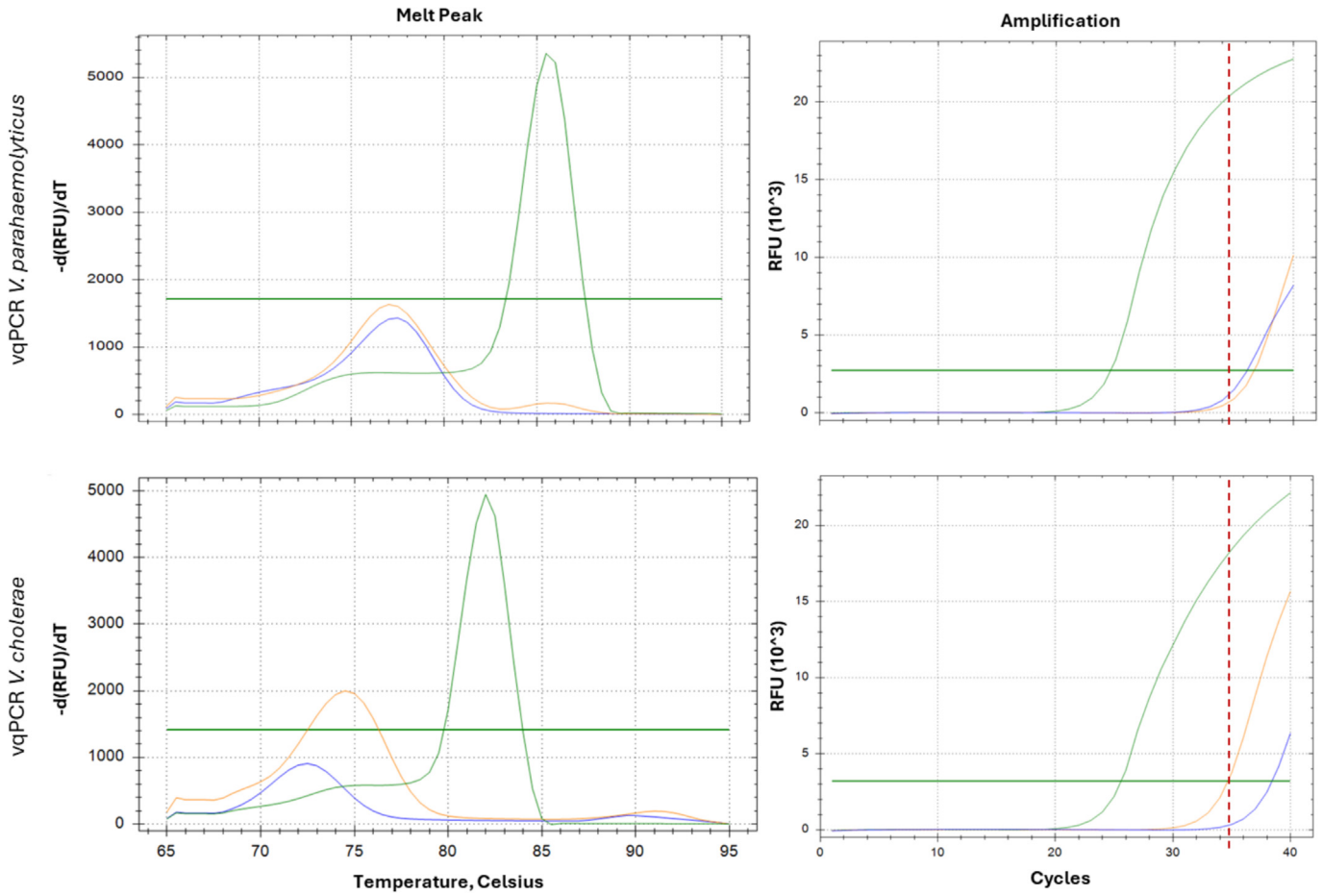

Figure 3. Melting peaks and amplification curves of vqPCR analysis of viable bacterial cells (green curves), heat-lysed dead bacterial cells (orange curves), and negative controls (blue curves) of *V. cholerae* and *V. parahaemolyticus*.

threshold were classified as dead cells (Robben et al., 2018). An intact cell membrane was observed for *V. parahaemolyticus* and *V. cholerae* VBNC cells in the LIVE/DEAD *BacLight* viability assay by green fluorescence, even if dead bacterial cells, which showed red fluorescence, were also detected in the LB suspension. An interesting finding was that the bacterial cells with an intact cell membrane showed morphological changes and formed a round shape instead of a rod shape. This morphological change has already been described for *Vibrio* spp. in the VBNC state (Falcioni et al., 2008). The induction of the VBNC state was additionally confirmed by the vqPCR assays and resulted in a Ct value of 29 (SD 0.2) for *V. parahaemolyticus* VBNC cells (6.2 Log10 cells/ml) and a Ct value of 28 (SD 0.3) for *V. cholerae* VBNC cells (6.8 Log10 cells/ml).

To sum up, a treatment of viable *V. parahaemolyticus* and *V. cholerae* cells in a combined solution of Lutensol A03 with 0.2 M $(NH_4)_2CO_3$ led to a reproducible VBNC induction of these bacteria (n = 9 biological replicates, each measured in triplicate). The VBNC state was confirmed by the absence of growth on LB medium, while metabolic activity and an intact cell membrane were detected in parallel, as described for bacteria in the VBNC state (Fleischmann et al., 2021).

Detection of only viable *V. parahaemolyticus* and *V. cholerae* including VBNC cells from food samples. To test the established singleplex vqPCR for detecting solely viable *V. parahaemolyticus* and *V. cholerae* cells, including cells in VBNC state, 38 seafood samples (n = 34 shrimp and n = 4 mussel samples) were analyzed for *V. parahaemolyticus* and 36 (n = 31 shrimp and n = 5 mussel samples) for *V. cholerae*. Comparing culture−based qualitative and quantitative detection methods, higher detection rates of *V. parahaemolyticus* with 53% (n = 20) and of *V. cholerae* with 58% (n = 21) were achieved when food samples were analyzed using vqPCR (Table 1). When seafood samples were analyzed according to the DIN/EN/ISO 21872-1:2023-06, only 29% (n = 11) of the samples were positive for *V. parahaemolyticus* and 6% (n = 2) for *V. cholerae*. A quantitative detection via a 1:10 dilution of the seafood samples and a subsequent plating on TCBS agar plates led to the lowest detection rates of 5% (n = 2) for *V. parahaemolyticus* and 3% (n = 1) for *V. cholerae*.

Overall, it was possible to detect up to 50% (n = 18) of samples positive for *V. parahaemolyticus* and 56% (n = 20) for *V. cholerae* using vqPCR alone, which would have been recognized as false-negative using culture-based detection methods. This suggests that the detected viable bacterial cells could be cells in VBNC state. However, there were also false negative results using vqPCR, whereby a detection was achieved exclusively using culture-based detection methods, suggesting a bacterial number in the sample below the vqPCR detection limit of 3.5 *V. parahaemolyticus* (20 fg DNA) and 6.9 *V. cholerae* (30 fg DNA) cells. In total, up to 4% (n = 2) of the seafood samples have been tested by vqPCR as false negatives for *V. parahaemolyticus* and 3% (n = 1) for *V. cholerae*.

However, it was possible to detect contaminations of seafood samples with viable *V. parahaemolyticus* from 2.6–4.2 Log10 cells/g and *V. cholerae* from 2.8–5.2 Log10 cells/g using vqPCR. A difference in PCR performance among the seafood matrices investigated was not detectable, as both shrimp and mussels with comparable natural contamination levels of *V. parahaemolyticus* and *V. cholerae* were analyzed.

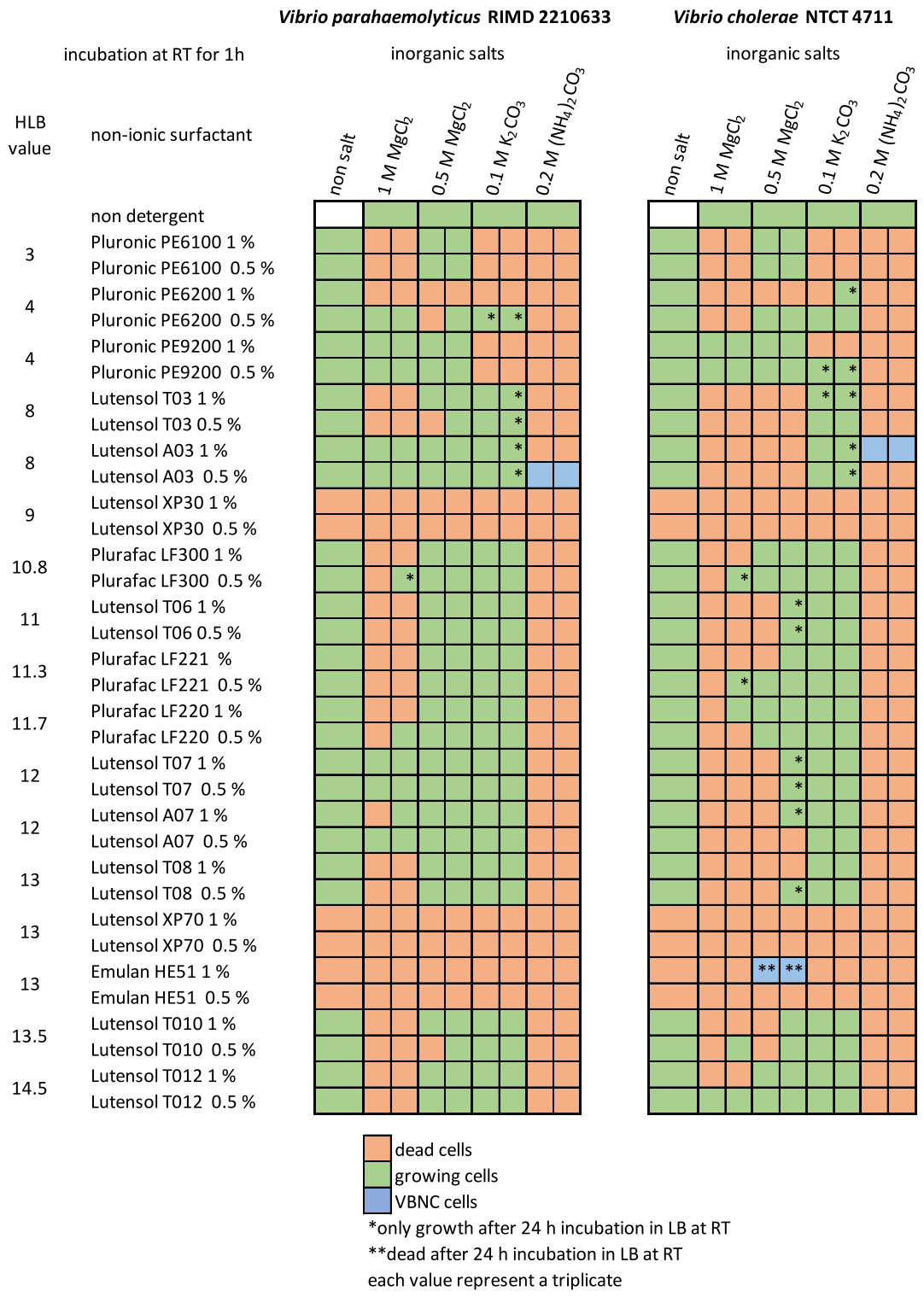

Figure 4. Screening of combined effects of nonionic surfactants and inorganic salts to induce the VBNC state in *V. parahaemolyticus* and *V. cholerae*. The results were obtained after an incubation time in a combined solution of nonionic surfactants and inorganic salts for 1 h at room temperature (RT) after washing the bacterial cells, and after a 24 h rest time in LB broth. The HLB value indicates the hydrophilic–lipophilic balance of the solutions combined. ***Combinations inducing stable VBNC cells, after 24 to 48 h (blue boxes), were carried out nine times.

Discussion

This study supports the VBNC state posing a problem in culture-based standard *Vibrio*-diagnostic according to ISO 21872-1:2023-06. Especially, the genus *Vibrio*, for which the VBNC state was first described (Xu et al., 1982), is known to enter into the VBNC state under unfavorable environmental and process conditions such as low temperatures during chilling and freezing (Di Salvo et al., 2023; Ramesh et al., 2024). Using the singleplex vqPCR established in this study to detect viable *V. parahaemolyticus* and *V. cholerae* cells, up to

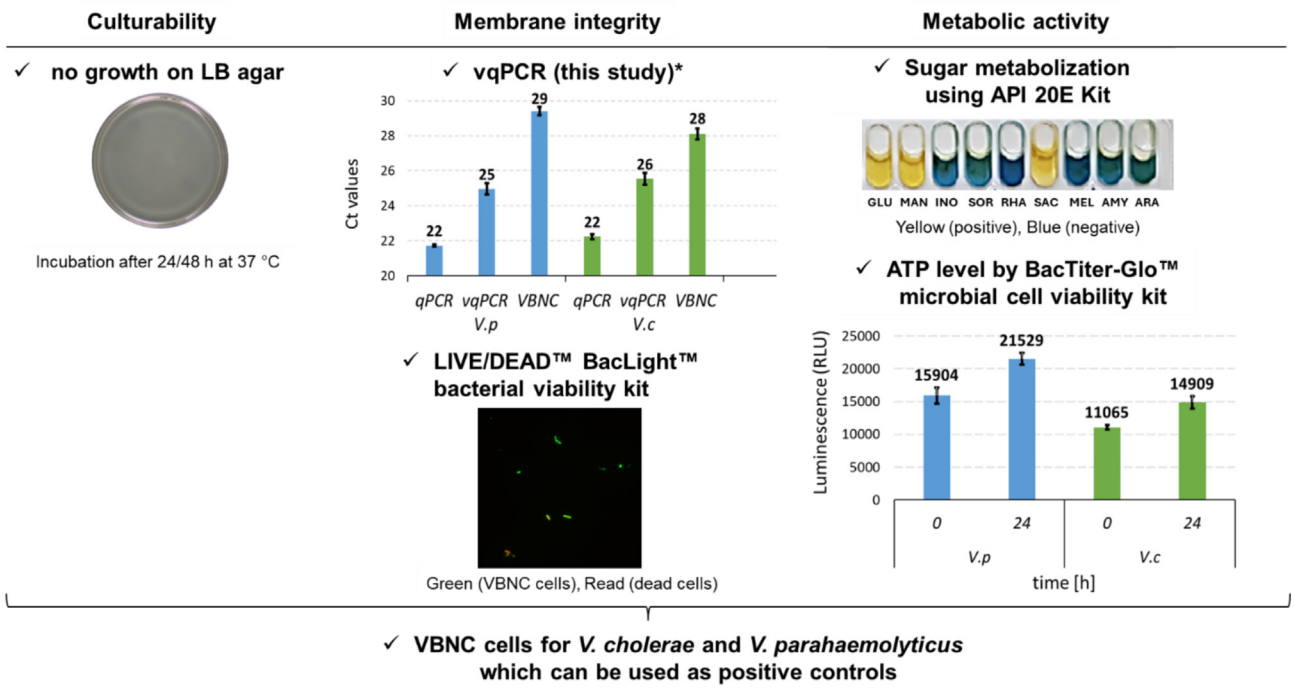

Figure 5. Summary of results on the confirmation of cells in the VBNC state using culture-based, membrane integrity, and metabolic activity assays. A combination of nonionic surfactants and inorganic salts was used to induce the VBNC state of culturable *V. parahaemolyticus* (*V.p*) and *V. cholerae* (*V.c*) cells. The results were summarized from nine independent experiments in which each sample was prepared in triplicate. *qPCR = total number of cells; vqPCR = number of viable cells; VBNC = number of induced VBNC cells.

Table 1
Comparison of culture – based quantitative and qualitative detection with vqPCR

Vibrio species	Number of samples	Qualitative detection using ISO 21872-1:2023-06*		Quantitative detection on TCBS agar*		Quantitative detection using vqPCR		Samples detected positive by vqPCR only	Samples detected false negative by vqPCR
		pos.	neg.	pos.	neg.	pos.	neg.		
V. p	38	29% (n = 11)	71% (n = 27)	5% (n = 2)	95% (n = 36)	53% (n = 20)	50% (n = 18)	50% (n = 18)	4% (n = 2)
V. c	36	6% (n = 2)	94% (n = 34)	3% (n = 1)	97% (n = 35)	58% (n = 21)	42% (n = 15)	56% (n = 20)	3% (n = 1)

V. p = *V. parahaemolyticus*; *V. c* = *V. cholerae*; pos. = positive; neg. = negative; Samples tested negative for *V. parahaemolyticus* and *V. cholerae,* with all methods were not included.

* Presumptive colonies were confirmed by conventional PCR according to ISO 21872-1:2023-06.

50% (n = 18) of samples were tested positive for *V. parahaemolyticus* and 56% (n = 20) for *V. cholerae* that would have been detected as false negatives by using culture-based detection methods, suggesting that the viable bacterial cells detected may be cells in the VBNC state. The detection of bacteria in the VBNC state is of great relevance regarding public health as these bacteria are not detectable by the DIN/EN/ISO 21872-1:2023-06 standard. However, they remain pathogenic due to their metabolic activity and toxin gene expression (Mishra, Taneja & Sharma, 2012) as well as their potential to return to vegetative growth under favorable environmental or host conditions. Culture-independent detection methods with high sensitivity are therefore becoming even more important. The established method described here achieved a very high detection sensitivity for *V. parahaemolyticus* with 20 fg DNA (3.5 bacterial cells) and for *V. cholerae* with 30 fg DNA (6.9 bacterial cells). However, the method also showed limitations, as false negative samples for *V. parahaemolyticus* (4%, n = 2) and *V. cholerae* (3%, n = 1) were also detected. Enrichment cultures and vqPCR diagnostics should therefore be performed in parallel to observe the detection limit of the vqPCR.

Previous studies on detecting VBNC cells via vqPCR approaches using DNA intercalating dyes are already published for *V. parahaemolyticus* (Zhu et al., 2012; Zhang et al., 2015; Niu et al., 2018; Yoon et al., 2019; Di Salvo et al., 2023) and *V. cholerae* (Wu, Liang & Kan, 2015). However, when establishing a well-functioning vqPCR protocol, it is important to consider the following aspects: (i) The principle of DNA intercalating dyes is based on the covalent binding of dyes to freely accessible DNA and DNA from membrane-compromised dead cells to inhibit their DNA amplification (Nocker, Cheung & Camper, 2006). Due to a low charge, ethidium monoazide (EMA) for example can also penetrate viable cells. Although metabolically active cells export EMA via transport pumps or diffusion barriers, the remaining chemical residues can lead to a substantial loss of DNA, resulting in false-negative results or inaccurate quantification. Furthermore, a study by Copin et al. (2021) described a toxic effect on *Vibrio* cells due to the ability of EMA to penetrate viable bacterial cells. Therefore, it is important to use strongly charged dyes such as propidium monoazide (PMA) or even Reagent D as described in this study. (ii) In addition to the DNA intercalating dye, the size of the tem-

plate is also important. Large target sequences up to 1600 bp ensure sufficient dye-DNA binding events happen regarding DNA from dead bacterial cells (Fittipaldi, Nocker & Codony, 2012). These events are necessary for the inhibition of DNA replication. With short target sequences between 100 and 200 bp, it is possible that only a few dye-DNA binding events occur on these short sequences, resulting in amplified target sequences of dead bacterial cells during PCR. Such misinterpretation of the vqPCR results could be possible in the studies by Zhu et al. (2012), Zhang et al. (2015) and Wu, Liang and Kan (2015) with target sequences of 233, 208, and 97 bp sequence length, respectively. However, the main disadvantage of long amplicons is the decrease in PCR efficiency due to a longer amplification time. For this purpose, intermediate – sized target gene sequences of 588 bp (*gro*EL) for *V. parahaemolyticus* (Hossain et al., 2012) and 510 bp (*omp*W) for *V. cholera*e (Nandi et al., 2000) were used in this study. These species-specific genetic markers ensure a comprehensive species-specific identification independent of individual virulence genes. Virulence genes such as *tdh, trh,* and *ctx* are not equally present in all strains of a species and are therefore unsuitable as universal diagnostic species-specific markers.

To establish a vqPCR assay, the inclusion of cells in VBNC state is challenging to ensure false positive or negative results. In all previous studies, either only viable and heat-killed *Vibrio* cells were used (Zhu et al., 2012; Zhang et al., 2015; Niu et al., 2018) and/or cells in VBNC state as control were generated by cold-starvation at 4 °C (Wu, Liang & Kan, 2015; Liu et al., 2018; Yoon et al., 2019; Di Salvo et al., 2023). The disadvantage of VBNC cells induced by cold-starvation is that this method requires at least up to 40 days for *V. parahaemolyticus* and 70 days for *V. cholerae* until solely VBNC state viable cells are present in the media (Wu, Liang & Kan, 2015; Liu et al., 2018). In this study, a rapid induction of VBNC cells of *V. parahaemolyticus* and *V. cholerae* within one hour was established according to the method by Robben et al. (2018). This method has been shown to be effective and reproducible in inducing the VBNC state in numerous bacterial species in a short time. A combined effect of Lutensol A03 and ammonium carbonate was found to be a reproducible (9 of 9 biological samples) combination resulting in VBNC state cells of *V. parahaemolyticus* and *V. cholerae,* which were stable for more than 48 h. During this time frame, VBNC cells may serve as reference controls for the development, standardization, and validation of detection methods for VBNC cells. However, during induction, a decrease in cell density was observed, resulting in 6.8 Log10 cells/ml (Ct 28) VBNC cells of *V. cholerae* and 6.2 Log10 cells/ml (Ct 29) VBNC cells of *V. parahaemolyticus* starting from a cell density of culturable bacterial cells of 7.3 Log10 cells/ml. However, differences of 1.1 Log for *V. parahaemolyticus* and 0.5 Log for *V. cholerae* were observed, which can be attributed to the methodology used. Nevertheless, a larger proportion of VBNC cells can be obtained via the VBNC induction with Lutensol A03 and ammonium carbonate, which can be used as a positive control for cells in the VBNC state, in contrast to the VBNC induction via cold-starvation. Liu et al. (2018) reported that after 40 days during cold-starvation of *V. parahaemolyticus* at 4 °C, a cell density of 4.8 Log10 cells/ml VBNC cells was observed from an original culture of culturable cells of 7.0 Log10 cells/ml.

In accordance with the findings of Liu et al. (2018), the integrity of the cell membrane was confirmed by fluorescent microscopy and metabolic activity investigation including ATP production. Another noteworthy observation was the morphological alteration of bacterial cells with intact cell membranes, which underwent a transformation from a rod-like shape, characteristic of *Vibrio* species, to a spherical shape. This morphological alteration has been previously noticed in numerous bacterial species (Kim et al., 2018).

Conclusion

In this study, two vqPCR methods were established to detect viable cells of *V. parahaemolyticus* and *V. cholerae*, including VBNC state cells. Using these vqPCR assays, up to 50% of samples tested in this study could be confirmed positive for *V. parahaemolyticus* and 56% for *V. cholerae*, which would have been recognized as false-negative by culture-based detection. Therefore, these vqPCR assays could be employed to ensure the food safety of these products and prevent infections caused by culturally undetected *V. parahaemolyticus* and *V. cholerae*. Quantification of viable *Vibrio* cells was achieved within three hours directly from seafood samples, allowing these assays to be used in parallel with culture-based tests to identify VBNC cells.

Simultaneously, a rapid and reproducible method was found to induce the VBNC state in *V. parahaemolyticus* and *V. cholerae*. The shift into the VBNC state was successfully achieved within one hour by treating cultivable cells with a combined solution of Lutensol A03 (0.5% for *V. parahaemolyticus* and 1.0% for *V. cholerae*) and ammonium carbonate (0.2 M). To our knowledge, such a rapid and reproducible induction of the VBNC state in *Vibrio* spp. has not been described before, offering the possibility to use these VBNC cells as controls for the development, standardization, and validation of VBNC detection methods.

CRediT authorship contribution statement

Eleonora Di Salvo: Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation. **Christopher Zeidler:** Writing – review & editing, Visualization, Investigation, Data curation. **Tim Bastian Schille:** Writing – review & editing, Visualization, Investigation, Data curation. **Patrick Mikuni-Mester:** Writing – review & editing, Conceptualization. **Thomas Alter:** Writing – review & editing, Conceptualization. **Stephan Huehn-Lindenbein:** Writing – review & editing, Conceptualization. **Susanne Fleischmann:** Writing – review & editing, Visualization, Supervision, Project administration, Investigation, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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