Adhesion to stainless steel surfaces and detection of viable but non cultivable cells of Vibrio parahaemolyticus and Vibrio cholerae isolated from shrimps in seafood processing environments: Stayin' alive?

Julia Mougin, Stéphanie Copin, Daline Bojolly, Virginie Raguenet, Annick Robert-Pillot, Marie-Laure Quilici, Graziella Midelet-Bourdin, Thierry Grard, Maryse Bonnin-Jusserand

Adhesion to stainless steel surfaces and detection of Viable But Non Cultivable cells of *Vibrio parahaemolyticus* and *Vibrio cholerae* isolated from shrimps in seafood processing environments: stayin' alive?

Julia Mougin[a,b,c,d,e,1], Stéphanie Copin[f,1], Daline Bojolly[a], Virginie Raguenet[f], Annick Robert-Pillot[g], Marie-Laure Quilici[g], Graziella Midelet-Bourdin[f], Thierry Grard[a*], Maryse Bonnin-Jusserand[a]

[a] *Univ. Littoral Côte d'Opale, convention ANSES, EA 7394 – ICV – Institut Charles Viollette, F-62321 Boulogne-sur-Mer, France*

[b] *INRA, France*

[c] *Univ. Lille F – 59000 Lille, France*

[d] *ISA, F – 59000 Lille, France*

[e] *Univ. Artois, F – 62000 Arras, France*

[f] *French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Laboratory for Food Safety, Boulevard du Bassin Napoléon, 62200 Boulogne-sur-Mer, France*

[g] *Institut Pasteur, Paris, Unité des Bactéries Pathogènes Entériques, Centre National de Référence des Vibrions et du Choléra*

E-mail address of each author:

Julia Mougin: julia.mougin@univ-littoral.fr

Stéphanie Copin: stephanie.copin@anses.fr

Daline Bojolly: daline.bojolly@gmail.com

Virginie Raguenet: virginie.raguenet@anses.fr

Annick Robert-Pillot: annick.robert-pillot@pasteur.fr

Marie-Laure Quilici: marie-laure.quilici@pasteur.fr

Graziella Midelet-Bourdin: graziella.bourdin@anses.fr

Thierry Grard*: thierry.grard@univ-littoral.fr

Maryse Bonnin-Jusserand: maryse.bonnin@univ-littoral.fr

*Corresponding author: Thierry Grard

Tel: +33 3 21 99 25 08. E-mail address: thierry.grard@univ-littoral.fr

[1] J. Mougin and S. Copin share co-authorship of this article.

Key words

Vibrio parahaemolyticus; Vibrio cholerae; biofilm; shrimps; VBNC; PMA-qPCR; seafood industries

Abstract

A single strain of *Vibrio parahaemolyticus* and a single stain of *Vibrio cholerae* were isolated from shrimps sourced from seafood processing plants. The isolated strains were evaluated for their ability to adhere to stainless steel surfaces, under conditions encountered in seafood industries. In this way, the impact of different environmental factors such as temperature (8 °C or 37 °C) and culture media (tryptic soy broth (TSB) 2% NaCl, artificial sea water (ASW) or industrial brine) were studied. The viability of these strains was analyzed by three different methods: the enumeration of cultivable bacteria on agar media, and two PCR methods, i.e. a qPCR and a PMA-qPCR method in order to distinguish between viable and dead cells. The proportion of viable but non-cultivable (VBNC) cells was evaluated by comparing the results of propidium monoazide real-time PCR (PMA-qPCR) with the enumeration results. Cultivable bacteria were not detected in brine, regardless of the conditions tested. However, *V. cholerae* in the VBNC state was detected in brine at 8 °C until 48h of incubation. *V. cholerae* exhibited higher viability at 8 °C compared to 37 °C. *V. cholerae* also exhibited higher viability compared to *V. parahaemolyticus*. In contrast, for *V. parahaemolyticus*, temperature and media (except industrial brine) had little influence on cell viability and adhesion behavior.

1. Introduction

Seafood can be contaminated by Gram-negative bacteria of the genus *Vibrio* present in coastal areas. In particular, *Vibrio parahaemolyticus* and *Vibrio cholerae* are two species potentially pathogenic for humans (Robert-Pillot, Copin, Himber, Gay, & Quilici, 2014). *V. cholerae* can be divided into two major groups: the cholera-causing strains of serogroups O1 and O139, and

non-O1/non-O139 *V. cholerae*. The pathogenicity of the strains depends on virulence factors, the most important of which is the cholera toxin (CT) encoded by the *ctx*A and *ctx*B genes. The non-O1/non-O139 strains rarely possess these cholera toxin genes but may also cause human illness. *V. parahaemolyticus* has been implicated in more than 45% of all outbreaks of bacterial gastroenteritis worldwide, and this proportion is increasing with the emergence of pandemic strains spreading over large geographic areas (Nair, et al., 2007). Typical *V. parahaemolyticus* illness results in mild to moderate acute gastroenteritis. As foodborne pathogens, *V. parahaemolyticus* and *V. cholerae* are responsible for vibriosis outbreaks and represent a risk to consumer health, especially in case of ingestion of raw, undercooked or mishandled seafood (Bonnin-Jusserand, et al., 2017); (M. F. R. Mizan, Jahid, & Ha, 2015). The risk associated with consumption of crustaceans, such as shrimps is well-documented for *V. parahaemolyticus* in several studies (Zarei, Borujeni, Jamnejad, & Khezrzadeh, 2012); (Abdullah Sani, Ariyawansa, Babji, & Hashim, 2013); (Xu, et al., 2014) and for both *V. parahaemolyticus* and *V. cholerae* in others (Rosec, Causse, Cruz, Rauzier, & Carnat, 2012); (Yano, et al., 2014). *Vibrio* spp. can adopt specific survival strategies in harsh environments. They are found either free-living or associated with sediments, suspended particles and plankton (Thomas, Joseph, Raveendran, & Nair, 2006); (Vezzulli, et al., 2010); (Vezzulli, Colwell, & Pruzzo, 2013)). Their ability to adhere to the surface of the exoskeleton of animals composed of chitin, some zooplankton organisms, especially copepods (Zidour, et al., 2017), and to plastic marine debris (Zettler, Mincer, & Amaral-Zettler, 2013) allows them to survive in seawater longer than in a free-living state. *Vibrio* spp. have the ability to form organized structures called biofilms on various biotic and abiotic surfaces (Watnick & Kolter, 1999). It has been shown that *Vibrio* spp. are able to form biofilms on aquaculture equipment such as larval rearing tanks (Bourne, Høj, Webster, Swan, & Hall, 2006) as well as on several other substrates, including glass, polystyrene, polyethylene and polyvinyl chloride (Snoussi, et al.,

2009). *Vibrio* spp. biofilms have also been detected on fishing industry equipment (Bagge-Ravn, et al., 2003). Furthermore, biofilms represent a well-known hazard in the food industry (Shi & Zhu, 2009); (Bridier, et al., 2015), and many environmental factors influence biofilm formation. For example, recent studies on the role of additives currently used in seafood processing, like NaCl and glucose, on the biofilm formation of *V. parahaemolyticus* on shrimp surfaces have been carried out (M. F. Mizan, et al., 2016). Within the biofilm, cells persist in different physiological stages, including in the viable but non cultivable (VBNC) state. The VBNC state is defined as a form of resistance of bacteria to adverse environmental conditions. The cells conserve their metabolic activity but cultivability is lost. Therefore, bacteria cannot be detected on agar Petri dishes (Oliver & Bockian, 1995). *V. parahaemolyticus* (H. Zhong, et al., 2017); (Su, Jane, & Wong, 2013); (Mizunoe, Wai, Ishikawa, Takade, & Yoshida, 2000) and *V. cholerae* (Rowan, 2004) can enter the VBNC state. Importantly, the ISO standard 21872-1 applied to detect potentially pathogenic *Vibrio* in seafood cannot detect bacteria in the VBNC state. This could lead to underestimation of the bacterial population.

This study focused on the biofilm-forming ability on stainless steel of two different potentially pathogenic for humans *Vibrio* species (*V. cholerae* and *V. parahaemolyticus*) isolated from shrimps. The processing of shrimps in the seafood industry follows different steps (GBPH, 2016). Considering the possibility of a recontamination risk after cooking, brine could play a role by transporting *Vibrio*. Interestingly, there is no further critical control point (CCP) making it possible to limit the microbial population level after this step. Moreover, brine is recycled. Therefore, pathogen or spoilage flora could be present for instance in the VBNC state if renewal is not sufficiently frequent. VBNC cells could hardly be detected by classical microbiological methods and thus represent a potential risk for consumers. In order to estimate the VBNC proportion of *V. parahaemolyticus* and *V. cholerae* within a biofilm

closest to industrial conditions, in our study, the experiments required the comparison of three different techniques: qPCR, PMA-qPCR (propidium monoazide real-time PCR) ((Q. Zhong, Tian, Wang, Fang, & Liao, 2018); (Zhu, Li, Jia, & Song, 2012); (Wu, Liang, & Kan, 2015)) and enumeration on agar plates. The influence of environmental parameters such as temperature and media, including industrial brine, has been assessed. The aim of this study consists of assessing the adhesion to stainless steel surfaces and detecting Viable But Non Cultivable cells of *Vibrio parahaemolyticus* and *Vibrio cholerae* isolated from shrimps in seafood processing environments.

2. Materials and methods

2.1 Bacterial strains and culture conditions

Two environmental strains, isolated by the National Reference Laboratory (NRL: *Vibrio* sp. in fishery products), ANSES Boulogne-sur-mer, from seafood industries, were used in this study. The first one, *Vibrio parahaemolyticus* (ANSES collection 14-B3PA-0046) was isolated from shrimps imported from Nigeria. The second one, *Vibrio cholerae* (ANSES collection 09-B3PA-2350) was isolated from shrimps imported from Madagascar. The strains were maintained in marine broth with 10% glycerol and stored at - 80 °C. Prior to the experiments, bacteria were initially streaked on nutrient agar supplemented with 2% NaCl and incubated overnight at 37 °C. The strains were then transferred into tryptic soy broth (TSB 2% NaCl) and incubated overnight at 37 °C.

Subsequently, cells were centrifuged at 5,000 *g* for 10 min and resuspended in TSB 2% NaCl or artificial sea water (ASW) or industrial brine (4 m^3 brine bath composed of water with a salinity of 1.17 ± 0.02 (>1.10), pH 4). This step was repeated twice. Bacterial cell concentrations were adjusted to 10^8 CFU/mL by spectrophotometry (LibraS11, BioChrom Ltd., Cambridge, UK). In order to check the bacterial concentrations, suitable dilutions of suspensions were made and plated on salted nutrient agar using an automatic spiral plater (easySpiral®, Interscience, St-Nom-la-Bretèche, France). The plates were incubated at 37 °C

for 24 h. Cell numbers were then quantified using an automatic colony counter (Scan500®, Interscience).

2.2 Preparation of 316 2B stainless steel coupons

AISI 316 2B (Fe/Cr18/Ni10/Mo3) stainless steel coupons (37x16x1 mm) were purchased from Goodfellow SARL (Lille, France). First of all, 316 2B stainless steel coupons were rubbed with paper, soaked in a 50% acetone/50% ethanol (v/v) solution. Coupons were then immersed in a 20% alkali detergent solution (v/v) (RBS_{50}, cleaning agent; Chemical Products R. Borghgraef, Brussels, Belgium) at 50 °C under agitation for 10 min. Coupons were rinsed in a 50 °C water bath under agitation for 20 min. Five successive rinses of 1 min each were carried out in ultra-pure water, while stirring at room temperature. Coupons were autoclaved for 15 min, at 121 °C.

2.3 Biofilm formation on stainless steel coupons

The bacterial suspension (11 mL) was transferred to a small sterile Petri dish (67 mm x 15 mm) containing a sterile coupon. In order to create a humid atmosphere, the Petri dishes were then placed in bigger Petri dishes (140 mm x 20 mm) containing paper towels soaked with 25 mL of sterilized distilled water. Sterile medium was used as a negative control. The Petri dishes were inoculated at 8 °C or 37 °C for 3 h, 24 h and 48 h.

2.4 Quantification of viable, VBNC and dead cells attached to stainless steel coupons after 3 h, 24 h and 48 h of incubation at either 37 °C or 8 °C

After incubation, cell suspensions were removed and coupons were washed with 25 mL of sterile physiological water (0.9 % NaCl) in order to remove non-adhered bacteria. Washed coupons were then transferred to sterile Petri dishes.

Two coupons were used per experiment. Each coupon was swabbed twice with the same swab. This swab was vortexed into a tube containing 2 mL of physiological water. A second

swab, after undergoing the same process, was added to the same tube. This tube containing the two swabs was then vortexed for 20 sec. The contents of the tube were divided into three fractions. Each fraction underwent one of the following three techniques:

- First fraction: the proportion of viable cultivable bacteria was obtained by plating serial dilutions on salted nutrient agar using an automatic spiral plater (easySpiral®, Interscience). The plates were incubated at 37 °C for 24 h. Cell numbers were then quantified using an automatic colony counter (Scan500®, Interscience).

- Second fraction: qPCR was used to amplify DNA of viable, VBNC and dead bacteria. Genomic DNA from cell suspensions (495 µL) was extracted using the DNeasy® Blood & Tissue Kit 250 (Qiagen, Courtabœuf, France), following the manufacturer's instructions. Then, *V. parahaemolyticus* DNA was quantified by qPCR following the protocol described by Robert-Pillot, et al. (2014), with modifications. First at all, the LightCycler® 480 (Roche Diagnostics, France) was used for qPCR. The Qiagen Master Mix QuantiTect® Probe PCR Kit was used. This method was optimized for *V. cholerae*. Concentrations of reagents were adjusted to reach 4 mM Mg^{2+} (Qiagen) and 6% glycerol using the Master Mix Light Cycler® 480 Probes Master (Roche). The PCR cycling conditions were adjusted by including initial denaturation at 95 °C for 15 sec, followed by annealing at 61 °C for 1 min, extension at 72 °C for 15 sec.

- Third fraction: PMA-qPCR was used to amplify DNA of viable and VBNC bacteria. Propidium monoazide, PMA (Biotium Inc., Hayward, CA, USA) dissolved in ultra-pure water was added to 495 µL of cell suspension to reach a final concentration of 50 mM. Following an incubation period of 5 min in the dark, samples were light-exposed using a photo-activation system (PhAst Blue, GenIUL, Barcelona, Spain), with an intensity of 80 for 10 min. DNA from cell suspensions was extracted using the DNeasy® Blood & Tissue Kit 250 (Qiagen), following the manufacturer's instructions. Then, *V. parahaemolyticus* and *V. cholerae* DNA

was quantified by qPCR following the protocol described above for the second fraction. The genome equivalents (GEs) of the *V. parahaemolyticus* and *V. cholerae* strains were calculated based on a *V. parahaemolyticus* genome of $5.0.10^6$ bp (Makino, et al., 2003) and on a *V. cholerae* genome of $4.0.10^6$ bp (Heidelberg, et al., 2000).

2.5 Microscopy observation

A coupon was used to image biofilm samples on stainless steel. The biofilm was stained for 15 min in the dark with 200 µL taken from a LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Carlsbad, CA, USA). Residual LIVE/DEAD® BacLight™ solution was removed. Bacteria attached to the coupon were then examined in a wet state under an epifluorescence microscope (Imager.Z1, Zeiss, Marly-le-Roi, France) connected to a CCD camera (Axiocam - MRm, Zeiss) with apotome (x 40 magnification). A minimum of five observations were taken for each observed coupon.

2.6 Statistical analysis

Biofilm experiments were carried out in triplicate (three independent experiments). Data were analyzed by one-way analysis of variance (ANOVA) using R3.3.3 software (R Core Team, 2017). The ANOVA model was verified using a Shap-iro-Wilk normality test and Bartlett test of homogeneity of variances. If the normality and homogeneity of variances of distribution were not verified, a Kruskal-Wallis test was carried out, followed by a post hoc test. Post hoc tests were performed with Bonferroni correction and Fisher's least significant difference (LSD) criterion, using the agricolae 1.2-8 package (De Mendibru, 2017). The results were expressed as means ± standard error. Statistical significance was considered at $p<0.05$.

3. Results

Vibrio parahaemolyticus ANSES collection 14-B3PA-0046

Total population (viable cultivable, VBNC and dead bacteria; qPCR)

206 In TSB 2% NaCl and ASW broths, there was no impact of the incubation time at either 37 °C
207 (Fig. 1) or 8 °C (Fig. 1) on the total population.

208 *Viable cultivable and VBNC bacteria (PMA-qPCR)*

209 In TSB 2% NaCl and ASW broths, the same proportion of bacteria was observed for 3 h (Fig.
210 1.a, 1.b), 24 h (Fig. 1.c, 1.d) and 48 h (Fig. 1.e, 1.f) at either 37 °C or 8 °C.

211 No additional viable bacteria were detected in brine, regardless of the conditions (Fig. 1).

212 *Viable cultivable bacteria (enumeration on Petri dishes)*

213 A significant decrease in bacteria numbers was observed in TSB 2% NaCl broth depending on
214 biofilm formation kinetics (3 h, 24 h and 48 h), regardless of the temperature (p<0.05) (Fig.
215 1). However, in ASW broth, there were more adherent bacteria at time point 24 h (Fig. 1.c,
216 1.d) compared to 3 h (Fig. 1.a, 1.b) and 48 h (Fig. 1.e, 1.f), regardless of the temperature
217 (p<0.05). No viable cultivable bacteria were detected in brine at either 37 °C (Fig. 1) or 8 °C
218 (Fig. 1).

219 *Vibrio cholerae* ANSES collection 09-B3PA-2350

220 *Total population (viable cultivable, VBNC and dead bacteria; qPCR)*

221 In TSB 2% NaCl broth, the total population had decreased at 37 °C at time point 48 h
222 (p<0.05) (Fig. 2.e). Otherwise, the total population was almost equivalent at 8 °C at 3 h (Fig.
223 2.b), 24 h (Fig. 2.d) and 48 h (Fig. 2.f). In ASW broth, there were no differences regardless of
224 the incubation time or the temperature. In brine, equivalent bacteria proportions were detected
225 at 8 °C, regardless of the incubation time (Fig. 2.b, 2.d, 2.f). In contrast, bacterial populations
226 had decreased at time point 48 h compared to 3 h and 24 h at 37 °C (p<0.05) (Fig. 2.a, 2.c,
227 2.e).

228 *Viable cultivable and VBNC bacteria (PMA-qPCR)*

229 In TSB 2% NaCl and ASW broths, the proportion of viable bacteria was equivalent regardless
230 of the incubation time and temperature (Fig. 2). In brine, bacteria in the VBNC state were

231 detected at 8 °C for each incubation time, including 48 h. (Fig. 2.b, 2.d, 2.f). This means that
232 the VBNC *V. cholerae* cells remained in brine at 8°C even for a long period of time.
233 However, at 37 °C, at time point 48 h, viable bacteria were no longer detected (Fig. 2.e).
234 Indeed, results showed no amplification curve of PMA-qPCR for an incubation time of 48 h
235 at 37 °C in brine only. Thus, all *V. cholerae* cells were dead (Fig. 2.e).

236 *Viable cultivable bacteria (enumeration on Petri dishes)*

237 In TSB 2% NaCl, the proportion of viable cultivable bacteria was not significant regardless of
238 the incubation time and the temperature (Fig. 2). In ASW, cultivable *V. cholerae* were not
239 detected at 37 °C regardless of the incubation time (Fig. 2.a, 2.c, 2.e). Furthermore, cultivable
240 *V. cholerae* were not detected at 8 °C at time point 3 h (Fig. 2.b), but the proportion of
241 bacteria was equivalent at 8 °C at 24 h (Fig. 2.d) and 48 h (Fig. 2.f) of incubation. No
242 additional viable cultivable *V. cholerae* were detected in brine, regardless of the incubation
243 time and temperature (Fig. 2).

244 *Comparisons between V. cholerae and V. parahaemolyticus in brine for an incubation time of*
245 *48 h*

246 In Brine, for an incubation time of 48 h, temperature had significant effect on the adhesion of
247 total bacteria regardless *Vibrio* species. Indeed, more bacteria were detected at 8 °C compared
248 to 37 °C (Fig. 3.1).

249 VBNC cells of *V. parahaemolyticus* were no longer detected either at 37 °C or 8 °C, while
250 VBNC cells of *V. cholerae* were detected at 8 °C only. According to these results, the
251 adhesion of VBNC cells could be strain-dependent. Moreover, these results demonstrated that
252 temperature has a significant effect on the adhesion of VBNC *V. cholerae* cells in brine (Fig.
253 3.2).

254 Viable cultivable bacteria were not detected either at 37 °C or 8 °C on Petri dishes (Fig. 3.3).

255 *Observations of adherent cells*

Micro-colonies were observed at 37 °C and 8 °C for *V. parahaemolyticus* at time points 3 h, 24 h and 48 h (Fig. 4). In brine, aggregates were observed from the early stages of adhesion (3 h), regardless of the temperature (Fig. 4.g, 4.p). These observations clearly showed a mix of live and dead bacteria during the establishment of these aggregates. Regarding *V. cholerae* (Fig. 5), dense cell clusters were observed from 3 h at 37 °C in TSB 2% NaCl (Fig. 5.a). These clusters decreased at 48 h, suggesting dispersal of the biofilm (Fig. 5.c). However, at 8 °C at time point 48 h (Fig. 5.l), clusters especially composed of dead cells were still present. In ASW broth, minor changes in the structure of the biofilm were observed, regardless of the temperature and the incubation time (Fig. 5.d, 5.e, 5.f, 5.m, 5.n, 5.o). In brine, small aggregates were observed regardless of the temperature and time (Fig. 5.g, 5.h, 5.i, 5.p, 5.q, 5.r). No further viable *V. cholerae* were observed in brine at 37 °C at time point 48 h (Fig. 5.i). Viable bacteria could be VBNC cells not detected by the molecular methods used or Petri dish enumeration. This could be explained by a detection threshold level of the techniques used that may be too low.

3. Discussion

This study provides a deeper understanding of the viability of bacteria inside the biofilm. qPCR and PMA-qPCR methods were developed for both *Vibrio* species, in addition to cell enumeration on agar. Importantly, the combined findings of these three techniques and their comparison provided the proportions of viable cultivable, VBNC and dead bacteria. Additionally, microscopy using a LIVE/DEAD® staining kit coupled with Apotome technology enabled us to supplement the results obtained by the methods mentioned above. In this way, we were able to observe bacterial adhesion on stainless steel coupons. These observations were corroborated by results obtained by enumeration on agar plates and molecular biology (qPCR and PMA-qPCR methods).

Many factors influence biofilm formation: the bacteria themselves, that is to say their own genetics and physiology, the type of attachment surface, and the environment (pH, temperature, nutrient availability) (Whitehead & Verran, 2015). Among these factors, temperature seems to play a key role in *Vibrio* biofilm formation. In this study, small differences were observed regarding *V. parahaemolyticus* and *V. cholerae* biofilms at either 37 °C or 8 °C, regardless of the incubation time (3 h, 24 h, 48 h). Indeed, the only significate influence of temperature determined was in brine but no influence was detected in TSB or ASW broths. Nonetheless, Ahmed, et al. (2018) showed that the biofilm formation of *V. parahaemolyticus* and *V. cholerae* was significantly higher at 37 °C and 25 °C than at 4 °C. In the study carried out by Beshiru and Igbinosa (2018), *V. cholerae* showed better abilities to form biofilm at 21 °C and 37 °C, while the highest biofilm production of *V. parahaemolyticus* was observed at 30 °C in TSB under dynamic culture. Similarly, Song, et al. (2017) observed better biofilm production of *V. parahaemolyticus* in TSB 3% NaCl at 25 °C compared to 15 °C and 37 °C. Han, Mizan, Jahid, and Ha (2016) showed that *V. parahaemolyticus* biofilm formation was better at 30 °C than 4 °C on stainless steel coupons immersed in fresh water. Furthermore, H.-C. Wong, Chung, and Yu (2002) showed that bacterial adhesion on stainless steel surfaces decreased when *V. parahaemolyticus* was incubated at 4 °C. All these results showed better biofilm formation for high temperatures (between 21 °C to 37 °C) compared to 4 °C, contrary to our findings.

Adhesion is also media-dependent. In this study, we did not observe the same behavior on either TSB 2 % NaCl or ASW between *Vibrio* spp. *V. parahaemolyticus* exhibited the same behavior in TSB or ASW, regardless of the incubation temperature. However, in ASW for 24 h and 48 h, better survival was observed for *V. cholerae* at 8 °C compared to 37 °C due to the presence of viable cultivable bacteria. The amount of viable cultivable bacteria at 37 °C in ASW was not higher than in TSB. H.-C. Wong, et al. (2002) showed that *V. parahaemolyticus*

exhibited better adhesion on stainless steel when cultured in chemically defined MM9 medium than TSB. Regarding *V. cholerae*, this behavior was dependent on the temperature of incubation. At 8 °C for 24 h and 48 h, there were no differences in adhesion on either TSB 2% NaCl or ASW. The fact that a nutrient-poor medium promotes adhesion has already been shown especially for *L. monocytogenes* (Kadam, et al., 2013). The composition of ASW medium is close to that of the marine environment, which is the natural habitat of *Vibrio*. Therefore, better biofilm formation ability could be expected in this medium, which was not the case in our study. Moreover, Beshiru and Igbinosa (2018) showed that *V. cholerae* and *V. parahaemolyticus* biofilm formation was not medium-dependent (even when the medium was rich or poor). Both tested strains also exhibited different behaviors in brine. Indeed, in this study, only dead bacteria were detected regardless of the temperature and incubation time for *V. parahaemolyticus*. However, VBNC bacteria of *V. cholerae* were detected at 37 °C for 3 h and 24 h. VBNC cells were detected for each incubation time at 8 °C. These results indicate a greater survival capacity of *V. cholerae* at 8 °C than at 37 °C in poor media. The induction of the VBNC state is triggered by a low temperature, more often 4 °C, and under starvation conditions (Q. Zhong, et al., 2018); (Su, et al., 2013); (H. C. Wong, Wang, Chen, & Chiu, 2004). This could explain the presence of VBNC cells of *V. cholerae* in brine at 8 °C.

As for temperature and media, bacterial adhesion depends on the food contact surface. Stainless steel coupons were used in this study to mimic the conditions encountered in seafood industries. Also, Han, et al. (2016) studied *V. parahaemolyticus* biofilm formation on stainless steel, crab and shrimp. In addition to surface hydrophoby, cell hydrophoby must also be taken into account: *Vibrio* are strongly hydrophilic (Beshiru & Igbinosa, 2018). However, it is difficult to compare studies of *Vibrio* biofilms with one another because, most of the time, biofilm formation was studied *via* the microtiter plate assay with crystal violet staining. This assay remains a useful screening tool, but does not reflect real biofilm formation

conditions; in particular, the surface type is not considered. Moreover, *Vibrio* biofilm formation is strain-dependent (Song, et al., 2017); (Odeyemi & Ahmad, 2017); (M. F. Mizan, et al., 2016); (H.-C. Wong, et al., 2002). To supplement our results, different *V. parahaemolyticus* and *V. cholerae* strains of different origins (isolated from different seafood products or different countries), including pathogenic strains (carrying pathogenicity factors such as *ctxA/ ctxB* genes for *V. cholerae* and *tdh* or *trh* genes for *V. parahaemolyticus*) should be assessed. Clearly, biofilm formation may be different depending on the strain's origin. The formation of mixed biofilm, for example *Vibrio* in association with *Listeria monocytogenes*, could also be evaluated. In addition to developing survival strategies (VBNC, biofilm), strains are constantly adapting to their environment. More and more studies point to the extent of antibiotic resistance for *Vibrio* (Yano, et al., 2014). This characteristic should therefore be taken into account in the biofilm survival state. Elexson, et al. (2014) tested several detergents against biofilms of antibiotic-resistant *V. parahaemolyticus* isolated from seafood and found hygiene issues and cross-contamination hazards in the seafood industries.

4. Conclusion

For the first time, the cell viability was taken into account in order to better understand the survival strategies of potentially pathogenic human *Vibrio*. The aim of this study was to get as close as possible to the conditions encountered in seafood industries, even if this test remained *in vitro* for the standardization and exploitation purposes of the results. This scoping study highlighted the behavior of both *Vibrio* strains under laboratory conditions for different environmental parameters. Despite the fact that there are few data on the risk for consumer health of *V. cholerae* from shrimps, the strain tested in this study showed a greater survival capacity than the *V. parahaemolyticus* strain. Indeed, VBNC *V. cholerae* cells were detected at 8 °C in brine for each incubation time, including 48 h. Therefore, it could be useful to monitor *V. cholerae* contamination in shrimps. Seafood processing companies need to be

aware of and take into account the *Vibrio* risk in their hazard analysis critical control point (HACCP) approach. The possible presence of VBNC cells cannot be ruled out. The use of molecular biology methods like PMA-qPCR is recommended to ensure the safety of seafood products.

Acknowledgements

Julia Mougin would like to thank the "Hauts-de-France" regional council for its financial support of her PhD studies. This work was funded by the French government and the region Hauts-de-France in the framework of the CPER 2014-2020 MARCO project. This work was supported by a grant from FranceAgriMer (2014-0813) as part of the SURVIB project. The authors also thank the CITPPM (*Confédération des industries de traitement des produits des pêches maritimes et de l'aquaculture*) as a contributor to the SURVIB project. The authors are also grateful to Thomas Brauge for his scientific assistance, to Cindy Ducrocq and Marie-Ange Huchin for technical assistance and to Alexandre Dehaut for advices in statistical analyses.

References

Abdullah Sani, N., Ariyawansa, S., Babji, A. S., & Hashim, J. K. (2013). The risk assessment of *Vibrio parahaemolyticus* in cooked black tiger shrimps (*Penaeus monodon*) in Malaysia. *Food control, 31*(2), 546-552.

Ahmed, H. A., El Bayomi, R. M., Hussein, M. A., Khedr, M. H. E., Abo Remela, E. M., & El-Ashram, A. M. M. (2018). Molecular characterization, antibiotic resistance pattern and biofilm formation of *Vibrio parahaemolyticus* and *V. cholerae* isolated from crustaceans and humans. *Int. J. Food Microbiol., 274*, 31-37.

Bagge-Ravn, D., Ng, Y., Hjelm, M., Christiansen, J. N., Johansen, C., & Gram, L. (2003). The microbial ecology of processing equipment in different fish industries - Analysis of the microflora during processing and following cleaning and disinfection. *Int. J. Food Microbiol., 87*(3), 239-250.

Beshiru, A., & Igbinosa, E. O. (2018). Characterization of extracellular virulence properties and biofilm-formation capacity of *Vibrio* species recovered from ready-to-eat (RTE) shrimps. *Microb. Pathog., 119*, 93-102.

Bonnin-Jusserand, M., Copin, S., Le Bris, C., Brauge, T., Gay, M., Brisabois, A., Grard, T., & Midelet-Bourdin, G. (2017). *Vibrio* species involved in seafood-borne outbreaks (*Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*): review of microbiological versus recent molecular detection methods in seafood products. *Crit. Rev. Food Sci. Nutr.*, 1-14.

Bourne, D. G., Høj, L., Webster, N. S., Swan, J., & Hall, M. R. (2006). Biofilm development within a larval rearing tank of the tropical rock lobster, *Panulirus ornatus*. *Aquaculture, 260*(1-4), 27-38.

391 Bridier, A., Sanchez-Vizuete, P., Guilbaud, M., Piard, J.-C., Naïtali, M., & Briandet, R. (2015).
392 Biofilm-associated persistence of food-borne pathogens. *Food Microbiol., 45*, 167-178.
393 De Mendibru, F. A. (2017). Statistical procedures for agricultural research. R package version
394 1.2-4. 2016. In.
395 Elexson, N., Afsah-Hejri, L., Rukayadi, Y., Soopna, P., Lee, H. Y., Tuan Zainazor, T. C., Nor Ainy,
396 M., Nakaguchi, Y., Mitsuaki, N., & Son, R. (2014). Effect of detergents as antibacterial
397 agents on biofilm of antibiotics-resistant *Vibrio parahaemolyticus* isolates. *Food control,*
398 *35*(1), 378-385.
399 GBPH. (2016). *Guides de bonnes pratiques d'hygiène : crustacés cuits* (Vol. 5961).
400 Han, N., Mizan, M. F. R., Jahid, I. K., & Ha, S.-D. (2016). Biofilm formation by *Vibrio*
401 *parahaemolyticus* on food and food contact surfaces increases with rise in temperature.
402 *Food control, 70*, 161-166.
403 Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D.
404 H., Hickey, E. K., Peterson, J. D., & Umayam, L. (2000). DNA sequence of both
405 chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature, 406*(6795), 477.
406 Kadam, S. R., den Besten, H. M., van der Veen, S., Zwietering, M. H., Moezelaar, R., & Abee, T.
407 (2013). Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of
408 growth condition, serotype and strain origin. *Int. J. Food Microbiol., 165*(3), 259-264.
409 Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima,
410 M., Nakano, M., Yamashita, A., Kubota, Y., Kimura, S., Yasunaga, T., Honda, T.,
411 Shinagawa, H., Hattori, M., & Iida, T. (2003). Genome sequence of *Vibrio*
412 *parahaemolyticus*: a pathogenic mechanism distinct from that of *V cholerae*. *Lancet,*
413 *361*(9359), 743-749.
414 Mizan, M. F., Jahid, I. K., Kim, M., Lee, K. H., Kim, T. J., & Ha, S. D. (2016). Variability in
415 biofilm formation correlates with hydrophobicity and quorum sensing among *Vibrio*
416 *parahaemolyticus* isolates from food contact surfaces and the distribution of the genes
417 involved in biofilm formation. *Biofouling, 32*(4), 497-509.
418 Mizan, M. F. R., Jahid, I. K., & Ha, S.-D. (2015). Microbial biofilms in seafood: A food-hygiene
419 challenge. *Food Microbiol., 49*, 41-55.
420 Mizunoe, Y., Wai, S. N., Ishikawa, T., Takade, A., & Yoshida, S.-i. (2000). Resuscitation of viable
421 but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under
422 starvation. *FEMS Microbiol. Lett., 186*(1), 115-120.
423 Nair, G. B., Ramamurthy, T., Bhattacharya, S. K., Dutta, B., Takeda, Y., & Sack, D. A. (2007).
424 Global dissemination of *Vibrio parahaemolyticus* serotype O3: K6 and its serovariants.
425 *Clin. Microbiol. Rev., 20*(1), 39-48.
426 Odeyemi, O. A., & Ahmad, A. (2017). Population dynamics, antibiotics resistance and biofilm
427 formation of *Aeromonas* and *Vibrio* species isolated from aquatic sources in Northern
428 Malaysia. *Microb. Pathog., 103*, 178-185.
429 Oliver, J. D., & Bockian, R. (1995). In vivo resuscitation, and virulence towards mice, of viable but
430 nonculturable cells of *Vibrio vulnificus*. *Appl. Environ. Microbiol., 61*(7), 2620-2623.
431 R Core Team. (2017). R: A language and environment for statistical computing (Version 3.4.
432 2)[Computer software]. *Vienna, Austria: R Foundation for Statistical Computing*.
433 Robert-Pillot, A., Copin, S., Himber, C., Gay, M., & Quilici, M.-L. (2014). Occurrence of the three
434 major *Vibrio* species pathogenic for human in seafood products consumed in France using
435 real-time PCR. *Int. J. Food Microbiol., 189*, 75-81.
436 Rosec, J.-P., Causse, V., Cruz, B., Rauzier, J., & Carnat, L. (2012). The international standard
437 ISO/TS 21872–1 to study the occurence of total and pathogenic *Vibrio parahaemolyticus*
438 and *Vibrio cholerae* in seafood: ITS improvement by use of a chromogenic medium and
439 PCR. *Int. J. Food Microbiol., 157*, 189-194.
440 Rowan, N. J. (2004). Viable but non-culturable forms of food and waterborne bacteria: Quo Vadis?
441 *Trends Food Sci. Technol., 15*(9), 462-467.
442 Shi, X., & Zhu, X. (2009). Biofilm formation and food safety in food industries. *Trends Food Sci.*
443 *Technol., 20*(9), 407-413.
444 Snoussi, M., Noumi, E., Hajlaoui, H., Usai, D., Sechi, L. A., Zanetti, S., & Bakhrouf, A. (2009).
445 High potential of adhesion to abiotic and biotic materials in fish aquaculture facility by
446 *Vibrio alginolyticus* strains. *J. Appl. Microbiol., 106*(5), 1591-1599.

Song, X., Ma, Y., Fu, J., Zhao, A., Guo, Z., Malakar, P. K., Pan, Y., & Zhao, Y. (2017). Effect of temperature on pathogenic and non-pathogenic *Vibrio parahaemolyticus* biofilm formation. *Food control, 73*, 485-491.

Su, C. P., Jane, W. N., & Wong, H. C. (2013). Changes of ultrastructure and stress tolerance of *Vibrio parahaemolyticus* upon entering viable but nonculturable state. *Int. J. Food Microbiol., 160*(3), 360-366.

Thomas, K. U., Joseph, N., Raveendran, O., & Nair, S. (2006). Salinity-induced survival strategy of *Vibrio cholerae* associated with copepods in Cochin backwaters. *Mar. Pollut. Bull., 52*(11), 1425-1430.

Vezzulli, L., Colwell, R. R., & Pruzzo, C. (2013). Ocean warming and spread of pathogenic *Vibrios* in the aquatic environment. *Microb. Ecol., 65*(4), 817-825.

Vezzulli, L., Previati, M., Pruzzo, C., Marchese, A., Bourne, D. G., & Cerrano, C. (2010). *Vibrio* infections triggering mass mortality events in a warming Mediterranean Sea. *Environ. Microbiol., 12*(7), 2007-2019.

Watnick, P. I., & Kolter, R. (1999). Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol. Microbiol., 34*(3), 586-595.

Whitehead, K. A., & Verran, J. (2015). Formation, architecture and functionality of microbial biofilms in the food industry. *Current Opinion in Food Science, 2*, 84-91.

Wong, H.-C., Chung, Y.-C., & Yu, J.-A. (2002). Attachment and inactivation of *Vibrio parahaemolyticus* on stainless steel and glass surface. *Food Microbiol., 19*(4), 341-350.

Wong, H. C., Wang, P., Chen, S. Y., & Chiu, S. W. (2004). Resuscitation of viable but non-culturable *Vibrio parahaemolyticus* in a minimum salt medium. *FEMS Microbiol. Lett., 233*(2), 269-275.

Wu, B., Liang, W., & Kan, B. (2015). Enumeration of viable non-culturable *Vibrio cholerae* using propidium monoazide combined with quantitative PCR. *J. Microbiol. Methods, 115*, 147-152.

Xu, X., Wu, Q., Zhang, J., Cheng, J., Zhang, S., & Wu, K. (2014). Prevalence, pathogenicity, and serotypes of *Vibrio parahaemolyticus* in shrimp from Chinese retail markets. *Food control, 46*, 81-85.

Yano, Y., Hamano, K., Satomi, M., Tsutsui, I., Ban, M., & Aue-umneoy, D. (2014). Prevalence and antimicrobial susceptibility of *Vibrio* species related to food safety isolated from shrimp cultured at inland ponds in Thailand. *Food control, 38*, 30-36.

Zarei, M., Borujeni, M. P., Jamnejad, A., & Khezrzadeh, M. (2012). Seasonal prevalence of *Vibrio* species in retail shrimps with an emphasis on *Vibrio parahaemolyticus*. *Food control, 25*, 107-109.

Zettler, E. R., Mincer, T. J., & Amaral-Zettler, L. A. (2013). Life in the "plastisphere": microbial communities on plastic marine debris. *Environ. Sci. Technol., 47*(13), 7137-7146.

Zhong, H., Zhong, Y., Deng, Q., Zhou, Z., Guan, X., Yan, M., Hu, T., & Luo, M. (2017). Virulence of thermolable haemolysi *tlh*, gastroenteritis related pathogenicity *tdh* and *trh* of the pathogens *Vibrio Parahemolyticus* in Viable but Non-Culturable (VBNC) state. *Microb. Pathog., 111*, 352-356.

Zhong, Q., Tian, J., Wang, J., Fang, X., & Liao, Z. (2018). iTRAQ-based proteomic analysis of the viable but nonculturable state of *Vibrio parahaemolyticus* ATCC 17802 induced by food preservative and low temperature. *Food control, 85*, 369-375.

Zhu, R. G., Li, T. P., Jia, Y. F., & Song, L. F. (2012). Quantitative study of viable *Vibrio parahaemolyticus* cells in raw seafood using propidium monoazide in combination with quantitative PCR. *J. Microbiol. Methods, 90*(3), 262-266.

Zidour, M., Chevalier, M., Belguesmia, Y., Cudennec, B., Grard, T., Drider, D., Souissi, S., & Flahaut, C. (2017). Isolation and characterization of bacteria colonizing *Acartia tonsa* copepod eggs and displaying antagonist effects against *Vibrio anguillarum*, *Vibrio alginolyticus* and other pathogenic strains. *Front. Microbiol., 8*(1919).

Figures caption:

Figure 1: *Vibrio parahaemolyticus* ANSES collection 14-B3PA-0046 at 37 °C (a,c,e) and 8 °C (b,d,f): enumeration of total bacteria: viable cultivable, VBNC, and dead bacteria (qPCR: black squares); viable cultivable and VBNC bacteria (PMA-qPCR: white squares); viable cultivable bacteria (on Petri dishes: grey squares) for 3 h, 24 h and 48 h of incubation on TSB (2% NaCl), ASW and brine media. Values are expressed by means, ± 95 % confidence interval, of three independent experiments.

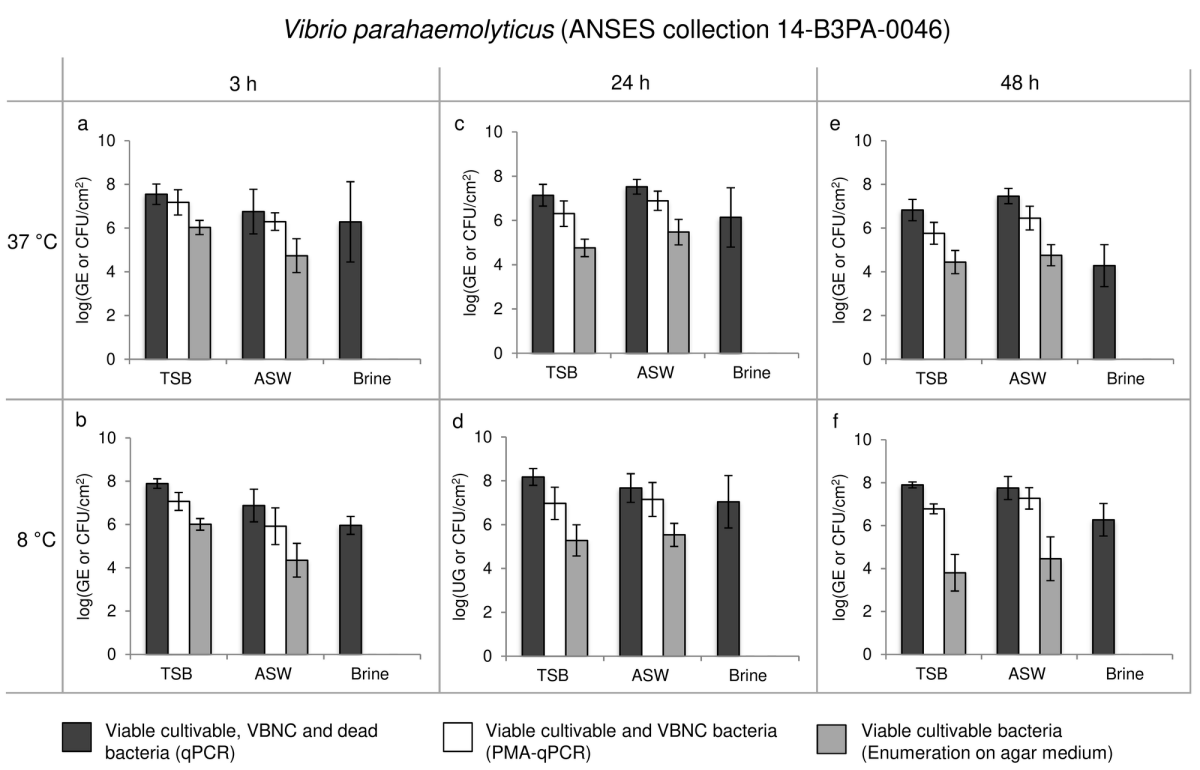
Figure 2: *Vibrio cholerae* ANSES collection 09-B3PA-2350 at 37 °C (a,c,e) and 8 °C (b,d,f): enumeration of total bacteria (qPCR: black squares); viable and VBNC bacteria (PMA-qPCR: white squares); viable cultivable bacteria (on Petri dishes: grey squares) for 3 h, 24 h and 48 h of incubation on TSB (2% NaCl), ASW and brine media. Values are expressed by means, ± 95 % confidence interval, of three independent experiments.

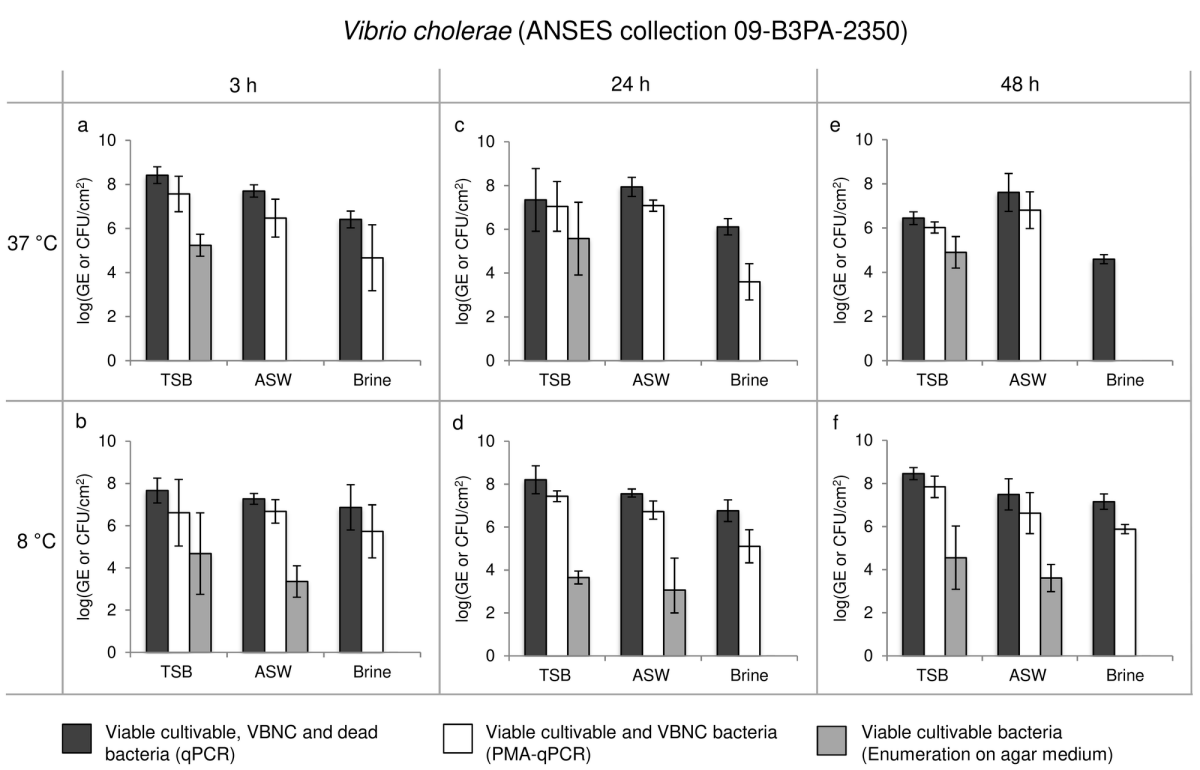
Figure 3: *Vibrio cholerae* ANSES collection 09-B3PA-2350 (hatched square) and *Vibrio parahaemolyticus* ANSES collection 14-B3PA-0046 (spotted square) in brine for 48 h, at 37 °C and 8 °C; Enumeration of (1) total bacteria (qPCR); (2) Viable and VBNC bacteria (PMA-qPCR); (3) Viable cultivable bacteria (on Petri dishes). Values are expressed by means, ± 95 % confidence interval, of three independent experiments. The letters a and b represent differences between treatments with two temperatures, according to post hoc tests. The "*" represents differences between strains at the same temperature, according to post hoc tests.

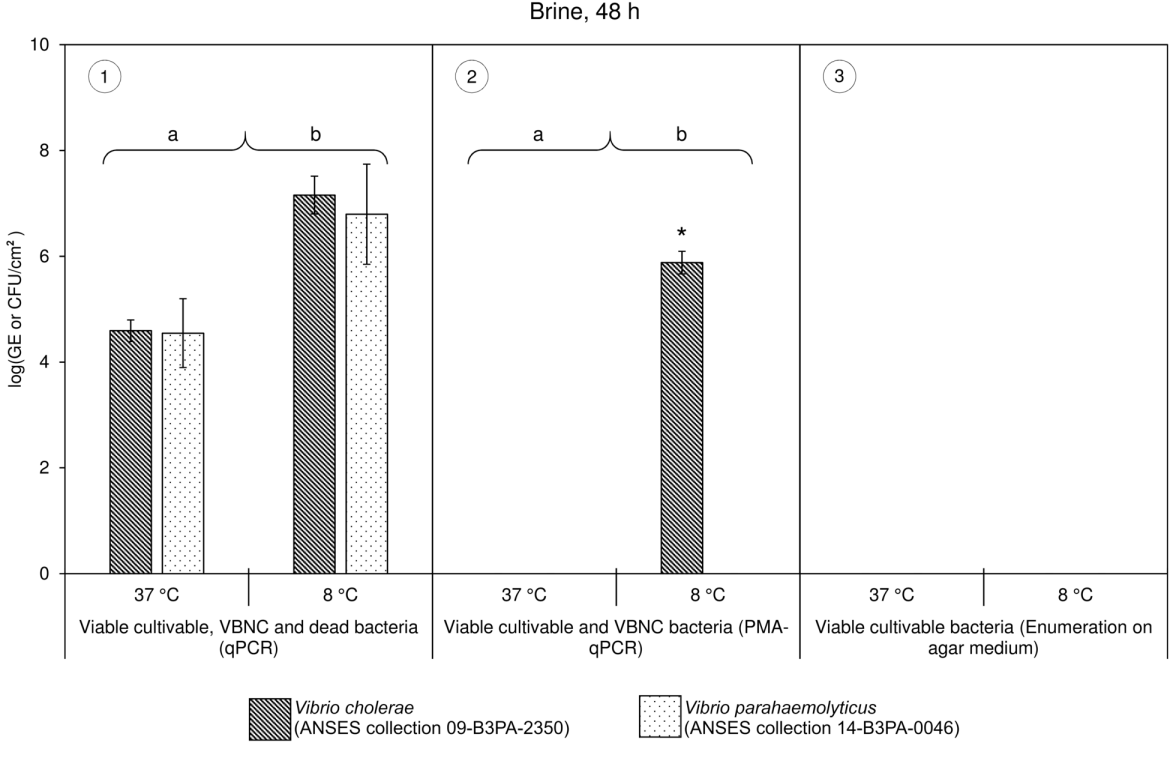
Figure 4: *Vibrio parahaemolyticus* ANSES collection 14-B3PA-0046 at 37 °C (a,b,c,d,e,f,g,h,i) and 8 °C (j,k,l,m,n,o,p,q,r): relevant microscopic photographs of stainless steel coupons after LIVE/DEAD®BacLight™ staining for 3 h, 24 h and 48 h of incubation on TSB (2% NaCl), ASW and brine media.

523 Experiments were repeated three times and a minimum of five images were taken for each
524 observed stainless steel coupon.

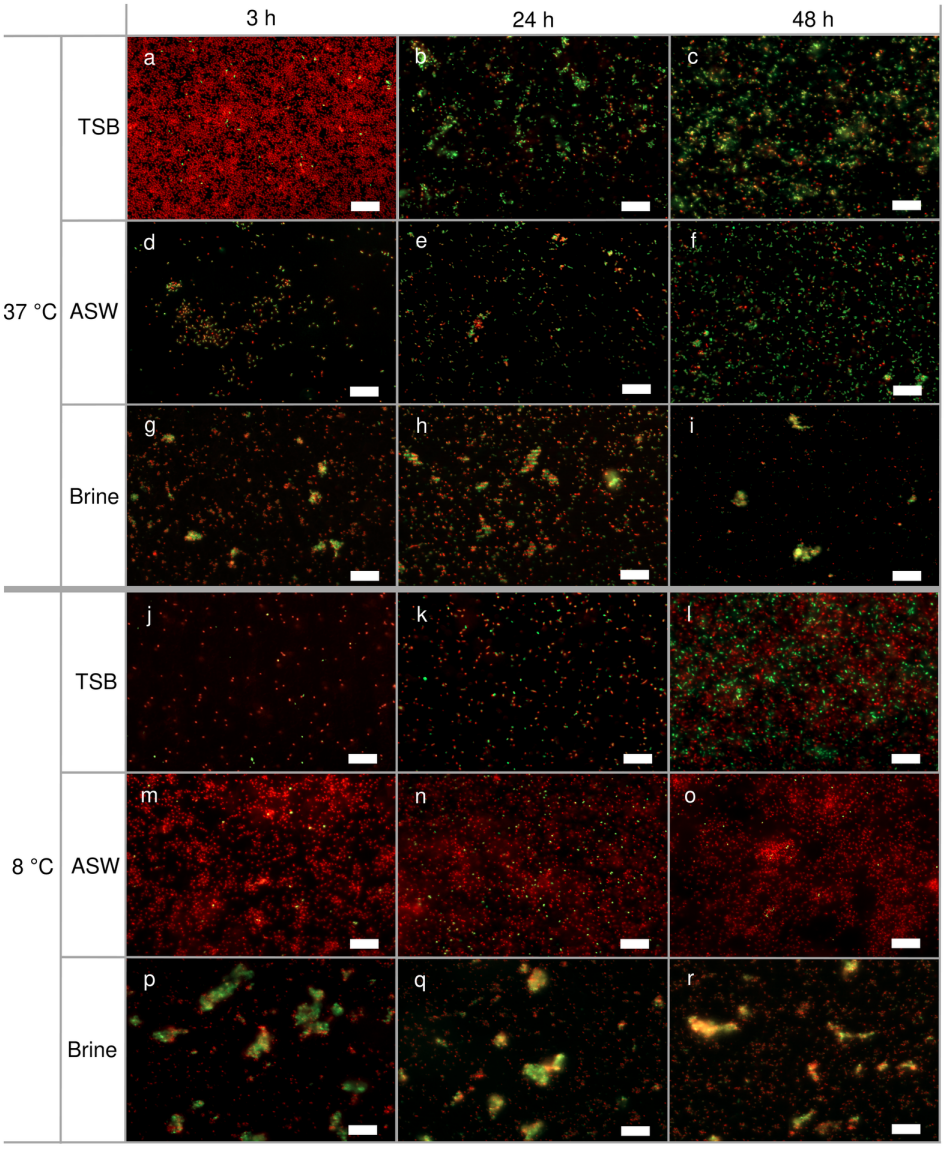
525 **Figure 5**: *Vibrio cholerae* ANSES collection 09-B3PA-2350 at 37 °C (a,b,c,d,e,f,g,h,i) and
526 8 °C (j,k,l,m,n,o,p,q,r): relevant microscopic photographs of stainless steel coupons after
527 LIVE/DEAD®BacLight^{TM} staining for 3 h, 24 h and 48 h of incubation on TSB (2% NaCl),
528 ASW and brine media.
529 Experiments were repeated three times and a minimum of five images were taken for each
530 observed stainless steel coupon.

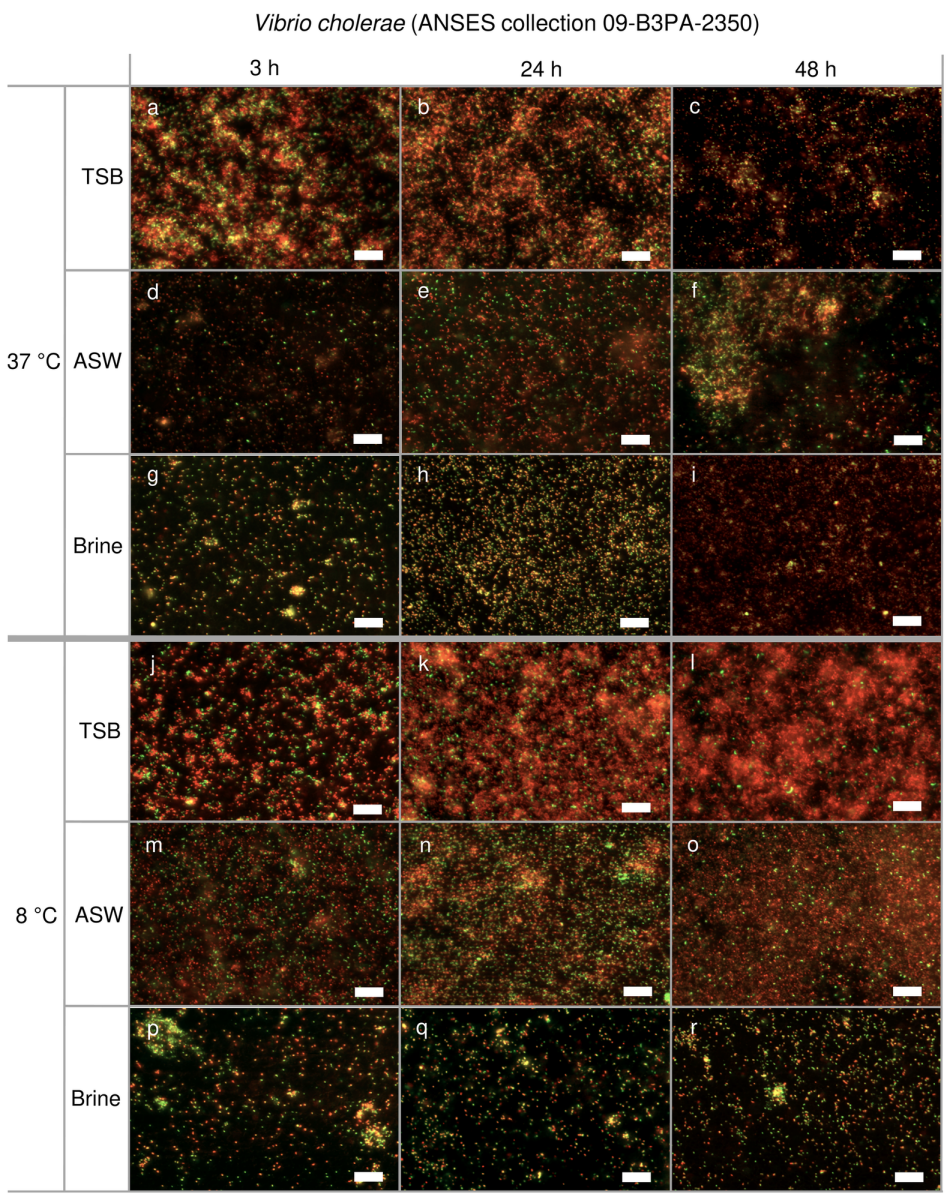

Vibrio parahaemolyticus (ANSES collection 14-B3PA-0046)
3 h
24 h
48 h
37 °C
8 °C
a
b
c
d
e
f
log(GE or CFU/cm²)
log(UG or CFU/cm²)
TSB
ASW
Brine
Viable cultivable, VBNC and dead bacteria (qPCR)
Viable cultivable and VBNC bacteria (PMA-qPCR)
Viable cultivable bacteria (Enumeration on agar medium)


Vibrio cholerae (ANSES collection 09-B3PA-2350)
3 h
24 h
48 h
37 °C
8 °C
a
b
c
d
e
f
log(GE or CFU/cm²)
0
2
4
6
8
10
TSB
ASW
Brine
Viable cultivable, VBNC and dead bacteria (qPCR)
Viable cultivable and VBNC bacteria (PMA-qPCR)
Viable cultivable bacteria (Enumeration on agar medium)


Brine, 48 h
log(GE or CFU/cm²)
10
8
6
4
2
0
1
2
3
a
b
a
b
*
37 °C
8 °C
37 °C
8 °C
37 °C
8 °C
Viable cultivable, VBNC and dead bacteria (qPCR)
Viable cultivable and VBNC bacteria (PMA-qPCR)
Viable cultivable bacteria (Enumeration on agar medium)
Vibrio cholerae (ANSES collection 09-B3PA-2350)
Vibrio parahaemolyticus (ANSES collection 14-B3PA-0046)

Vibrio parahaemolyticus (ANSES collection 14-B3PA-0046)




Vibrio cholerae (ANSES collection 09-B3PA-2350)
3 h
24 h
48 h
37 °C
8 °C
TSB
ASW
Brine
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r