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

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32 **Key words**

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

35 **Abstract**

36 A single strain of *Vibrio parahaemolyticus* and a single stain of *Vibrio cholerae* were isolated
37 from shrimps sourced from seafood processing plants. The isolated strains were evaluated for
38 their ability to adhere to stainless steel surfaces, under conditions encountered in seafood
39 industries. In this way, the impact of different environmental factors such as temperature
40 (8 °C or 37 °C) and culture media (tryptic soy broth (TSB) 2% NaCl, artificial sea water
41 (ASW) or industrial brine) were studied. The viability of these strains was analyzed by three
42 different methods: the enumeration of cultivable bacteria on agar media, and two PCR
43 methods, i.e. a qPCR and a PMA-qPCR method in order to distinguish between viable and
44 dead cells. The proportion of viable but non-cultivable (VBNC) cells was evaluated by
45 comparing the results of propidium monoazide real-time PCR (PMA-qPCR) with the
46 enumeration results. Cultivable bacteria were not detected in brine, regardless of the
47 conditions tested. However, *V. cholerae* in the VBNC state was detected in brine at 8 °C until
48 48h of incubation. *V. cholerae* exhibited higher viability at 8 °C compared to 37 °C. *V.*
49 *cholerae* also exhibited higher viability compared to *V. parahaemolyticus*. In contrast, for *V.*
50 *parahaemolyticus*, temperature and media (except industrial brine) had little influence on cell
51 viability and adhesion behavior.

52 **1. Introduction**

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

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

82 2009). *Vibrio* spp. biofilms have also been detected on fishing industry equipment (Bagge-
83 Ravn, et al., 2003). Furthermore, biofilms represent a well-known hazard in the food industry
84 (Shi & Zhu, 2009); (Bridier, et al., 2015), and many environmental factors influence biofilm
85 formation. For example, recent studies on the role of additives currently used in seafood
86 processing, like NaCl and glucose, on the biofilm formation of *V. parahaemolyticus* on
87 shrimp surfaces have been carried out (M. F. Mizan, et al., 2016). Within the biofilm, cells
88 persist in different physiological stages, including in the viable but non cultivable (VBNC)
89 state. The VBNC state is defined as a form of resistance of bacteria to adverse environmental
90 conditions. The cells conserve their metabolic activity but cultivability is lost. Therefore,
91 bacteria cannot be detected on agar Petri dishes (Oliver & Bockian, 1995). *V.*
92 *parahaemolyticus* (H. Zhong, et al., 2017); (Su, Jane, & Wong, 2013); (Mizunoe, Wai,
93 Ishikawa, Takade, & Yoshida, 2000) and *V. cholerae* (Rowan, 2004) can enter the VBNC
94 state. Importantly, the ISO standard 21872-1 applied to detect potentially pathogenic *Vibrio* in
95 seafood cannot detect bacteria in the VBNC state. This could lead to underestimation of the
96 bacterial population.

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

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

115 **2. Materials and methods**

116 **2.1 Bacterial strains and culture conditions**

117 Two environmental strains, isolated by the National Reference Laboratory (NRL: *Vibrio* sp.
118 in fishery products), ANSES Boulogne-sur-mer, from seafood industries, were used in this
119 study. The first one, *Vibrio parahaemolyticus* (ANSES collection 14-B3PA-0046) was
120 isolated from shrimps imported from Nigeria. The second one, *Vibrio cholerae* (ANSES
121 collection 09-B3PA-2350) was isolated from shrimps imported from Madagascar. The strains
122 were maintained in marine broth with 10% glycerol and stored at - 80 °C. Prior to the
123 experiments, bacteria were initially streaked on nutrient agar supplemented with 2% NaCl and
124 incubated overnight at 37 °C. The strains were then transferred into tryptic soy broth (TSB
125 2% NaCl) and incubated overnight at 37 °C.

126 Subsequently, cells were centrifuged at 5,000 g for 10 min and resuspended in TSB 2% NaCl
127 or artificial sea water (ASW) or industrial brine (4 m³ brine bath composed of water with a
128 salinity of 1.17 ± 0.02 (>1.10), pH 4). This step was repeated twice. Bacterial cell
129 concentrations were adjusted to 10⁸ CFU/mL by spectrophotometry (LibraS11, BioChrom
130 Ltd., Cambridge, UK). In order to check the bacterial concentrations, suitable dilutions of
131 suspensions were made and plated on salted nutrient agar using an automatic spiral plater
132 (easySpiral[®], Interscience, St-Nom-la-Bretèche, France). The plates were incubated at 37 °C

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

135 **2.2 Preparation of 316 2B stainless steel coupons**

136 AISI 316 2B (Fe/Cr18/Ni10/Mo3) stainless steel coupons (37x16x1 mm) were purchased
137 from Goodfellow SARL (Lille, France). First of all, 316 2B stainless steel coupons were
138 rubbed with paper, soaked in a 50% acetone/50% ethanol (v/v) solution. Coupons were then
139 immersed in a 20% alkali detergent solution (v/v) (RBS₅₀, cleaning agent; Chemical Products
140 R. Borghgraef, Brussels, Belgium) at 50 °C under agitation for 10 min. Coupons were rinsed
141 in a 50 °C water bath under agitation for 20 min. Five successive rinses of 1 min each were
142 carried out in ultra-pure water, while stirring at room temperature. Coupons were autoclaved
143 for 15 min, at 121 °C.

144 **2.3 Biofilm formation on stainless steel coupons**

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

150 **2.4 Quantification of viable, VBNC and dead cells attached to stainless steel coupons**

151 **after 3 h, 24 h and 48 h of incubation at either 37 °C or 8 °C**

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

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

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

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

164 - Second fraction: qPCR was used to amplify DNA of viable, VBNC and dead bacteria.
165 Genomic DNA from cell suspensions (495 µL) was extracted using the DNeasy[®] Blood &
166 Tissue Kit 250 (Qiagen, Courtabœuf, France), following the manufacturer's instructions.
167 Then, *V. parahaemolyticus* DNA was quantified by qPCR following the protocol described by
168 Robert-Pillot, et al. (2014), with modifications. First at all, the LightCycler[®] 480 (Roche
169 Diagnostics, France) was used for qPCR. The Qiagen Master Mix QuantiTect[®] Probe PCR Kit
170 was used. This method was optimized for *V. cholerae*. Concentrations of reagents were
171 adjusted to reach 4 mM Mg²⁺ (Qiagen) and 6% glycerol using the Master Mix Light Cycler[®]
172 480 Probes Master (Roche). The PCR cycling conditions were adjusted by including initial
173 denaturation at 95 °C for 15 sec, followed by annealing at 61 °C for 1 min, extension at 72 °C
174 for 15 sec.

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

182 was quantified by qPCR following the protocol described above for the second fraction. The
183 genome equivalents (GEs) of the *V. parahaemolyticus* and *V. cholerae* strains were calculated
184 based on a *V. parahaemolyticus* genome of $5.0 \cdot 10^6$ bp (Makino, et al., 2003) and on a *V.*
185 *cholerae* genome of $4.0 \cdot 10^6$ bp (Heidelberg, et al., 2000).

186 **2.5 Microscopy observation**

187 A coupon was used to image biofilm samples on stainless steel. The biofilm was stained for
188 15 min in the dark with 200 μ L taken from a LIVE/DEAD[®] BacLight[™] bacterial viability kit
189 (Invitrogen, Carlsbad, CA, USA). Residual LIVE/DEAD[®] BacLight[™] solution was removed.
190 Bacteria attached to the coupon were then examined in a wet state under an epifluorescence
191 microscope (Imager.Z1, Zeiss, Marly-le-Roi, France) connected to a CCD camera (Axiocam -
192 MRm, Zeiss) with apotome (x 40 magnification). A minimum of five observations were taken
193 for each observed coupon.

194 **2.6 Statistical analysis**

195 Biofilm experiments were carried out in triplicate (three independent experiments). Data were
196 analyzed by one-way analysis of variance (ANOVA) using R3.3.3 software (R Core Team,
197 2017). The ANOVA model was verified using a Shapiro-Wilk normality test and Bartlett test
198 of homogeneity of variances. If the normality and homogeneity of variances of distribution
199 were not verified, a Kruskal-Wallis test was carried out, followed by a post hoc test. Post hoc
200 tests were performed with Bonferroni correction and Fisher's least significant difference
201 (LSD) criterion, using the agricolae 1.2-8 package (De Mendibru, 2017). The results were
202 expressed as means \pm standard error. Statistical significance was considered at $p < 0.05$.

203 **3. Results**

204 *Vibrio parahaemolyticus* ANSES collection 14-B3PA-0046

205 *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*

256 Micro-colonies were observed at 37 °C and 8 °C for *V. parahaemolyticus* at time points 3 h,
257 24 h and 48 h (Fig. 4). In brine, aggregates were observed from the early stages of adhesion (3
258 h), regardless of the temperature (Fig. 4.g, 4.p). These observations clearly showed a mix of
259 live and dead bacteria during the establishment of these aggregates. Regarding *V. cholerae*
260 (Fig. 5), dense cell clusters were observed from 3 h at 37 °C in TSB 2% NaCl (Fig. 5.a).
261 These clusters decreased at 48 h, suggesting dispersal of the biofilm (Fig. 5.c). However, at
262 8 °C at time point 48 h (Fig. 5.l), clusters especially composed of dead cells were still present.
263 In ASW broth, minor changes in the structure of the biofilm were observed, regardless of the
264 temperature and the incubation time (Fig. 5.d, 5.e, 5.f, 5.m, 5.n, 5.o). In brine, small
265 aggregates were observed regardless of the temperature and time (Fig. 5.g, 5.h, 5.i, 5.p, 5.q,
266 5.r). No further viable *V. cholerae* were observed in brine at 37 °C at time point 48 h (Fig.
267 5.i). Viable bacteria could be VBNC cells not detected by the molecular methods used or Petri
268 dish enumeration. This could be explained by a detection threshold level of the techniques
269 used that may be too low.

270 **3. Discussion**

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

280 Many factors influence biofilm formation: the bacteria themselves, that is to say their own
281 genetics and physiology, the type of attachment surface, and the environment (pH,
282 temperature, nutrient availability) (Whitehead & Verran, 2015). Among these factors,
283 temperature seems to play a key role in *Vibrio* biofilm formation. In this study, small
284 differences were observed regarding *V. parahaemolyticus* and *V. cholerae* biofilms at either
285 37 °C or 8 °C, regardless of the incubation time (3 h, 24 h, 48 h). Indeed, the only significant
286 influence of temperature determined was in brine but no influence was detected in TSB or
287 ASW broths. Nonetheless, Ahmed, et al. (2018) showed that the biofilm formation of *V.*
288 *parahaemolyticus* and *V. cholerae* was significantly higher at 37 °C and 25 °C than at 4 °C. In
289 the study carried out by Beshiru and Igbinsosa (2018), *V. cholerae* showed better abilities to
290 form biofilm at 21 °C and 37 °C, while the highest biofilm production of *V. parahaemolyticus*
291 was observed at 30 °C in TSB under dynamic culture. Similarly, Song, et al. (2017) observed
292 better biofilm production of *V. parahaemolyticus* in TSB 3% NaCl at 25 °C compared to
293 15 °C and 37 °C. Han, Mizan, Jahid, and Ha (2016) showed that *V. parahaemolyticus* biofilm
294 formation was better at 30 °C than 4 °C on stainless steel coupons immersed in fresh water.
295 Furthermore, H.-C. Wong, Chung, and Yu (2002) showed that bacterial adhesion on stainless
296 steel surfaces decreased when *V. parahaemolyticus* was incubated at 4 °C. All these results
297 showed better biofilm formation for high temperatures (between 21 °C to 37 °C) compared to
298 4 °C, contrary to our findings.

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

305 exhibited better adhesion on stainless steel when cultured in chemically defined MM9
306 medium than TSB. Regarding *V. cholerae*, this behavior was dependent on the temperature of
307 incubation. At 8 °C for 24 h and 48 h, there were no differences in adhesion on either TSB
308 2% NaCl or ASW. The fact that a nutrient-poor medium promotes adhesion has already been
309 shown especially for *L. monocytogenes* (Kadam, et al., 2013). The composition of ASW
310 medium is close to that of the marine environment, which is the natural habitat of *Vibrio*.
311 Therefore, better biofilm formation ability could be expected in this medium, which was not
312 the case in our study. Moreover, Beshiru and Igbinosa (2018) showed that *V. cholerae* and *V.*
313 *parahaemolyticus* biofilm formation was not medium-dependent (even when the medium was
314 rich or poor). Both tested strains also exhibited different behaviors in brine. Indeed, in this
315 study, only dead bacteria were detected regardless of the temperature and incubation time for
316 *V. parahaemolyticus*. However, VBNC bacteria of *V. cholerae* were detected at 37 °C for 3 h
317 and 24 h. VBNC cells were detected for each incubation time at 8 °C. These results indicate a
318 greater survival capacity of *V. cholerae* at 8 °C than at 37 °C in poor media. The induction of
319 the VBNC state is triggered by a low temperature, more often 4 °C, and under starvation
320 conditions (Q. Zhong, et al., 2018); (Su, et al., 2013); (H. C. Wong, Wang, Chen, & Chiu,
321 2004). This could explain the presence of VBNC cells of *V. cholerae* in brine at 8 °C.

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

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

344 **4. Conclusion**

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

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

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- 498

499 **Figures caption:**

500 **Figure 1:** *Vibrio parahaemolyticus* ANSES collection 14-B3PA-0046 at 37 °C (a,c,e) and
501 8 °C (b,d,f): enumeration of total bacteria: viable cultivable, VBNC, and dead bacteria
502 (qPCR: black squares); viable cultivable and VBNC bacteria (PMA-qPCR: white squares);
503 viable cultivable bacteria (on Petri dishes: grey squares) for 3 h, 24 h and 48 h of incubation
504 on TSB (2% NaCl), ASW and brine media. Values are expressed by means, \pm 95 %
505 confidence interval, of three independent experiments.

506 **Figure 2:** *Vibrio cholerae* ANSES collection 09-B3PA-2350 at 37 °C (a,c,e) and 8 °C (b,d,f):
507 enumeration of total bacteria (qPCR: black squares); viable and VBNC bacteria (PMA-qPCR:
508 white squares); viable cultivable bacteria (on Petri dishes: grey squares) for 3 h, 24 h and 48 h
509 of incubation on TSB (2% NaCl), ASW and brine media. Values are expressed by means, \pm
510 95 % confidence interval, of three independent experiments.

511 **Figure 3:** *Vibrio cholerae* ANSES collection 09-B3PA-2350 (hatched square) and *Vibrio*
512 *parahaemolyticus* ANSES collection 14-B3PA-0046 (spotted square) in brine for 48 h, at
513 37 °C and 8 °C; Enumeration of (1) total bacteria (qPCR); (2) Viable and VBNC bacteria
514 (PMA-qPCR); (3) Viable cultivable bacteria (on Petri dishes). Values are expressed by
515 means, \pm 95 % confidence interval, of three independent experiments. The letters a and b
516 represent differences between treatments with two temperatures, according to post hoc tests.
517 The “*” represents differences between strains at the same temperature, according to post hoc
518 tests.

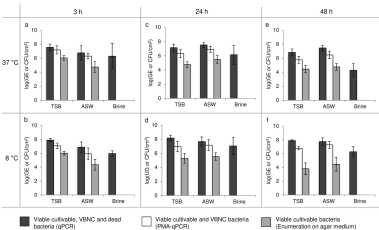
519 **Figure 4:** *Vibrio parahaemolyticus* ANSES collection 14-B3PA-0046 at 37 °C
520 (a,b,c,d,e,f,g,h,i) and 8 °C (j,k,l,m,n,o,p,q,r): relevant microscopic photographs of stainless
521 steel coupons after LIVE/DEAD[®]BacLight[™] staining for 3 h, 24 h and 48 h of incubation on
522 TSB (2% NaCl), ASW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

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

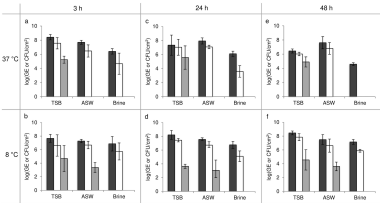
525 **Figure 5:** *Vibrio cholerae* ANSES collection 09-B3PA-2350 at 37 °C (a,b,c,d,e,f,g,h,i) and
526 8 °C (j,k,l,m,n,o,p,q,r): relevant microscopic photographs of stainless steel coupons after
527 LIVE/DEAD®BacLight™ staining for 3 h, 24 h and 48 h of incubation on TSB (2% NaCl),
528 ASW and brine 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)



Vibrio cholerae (ANSES collection 09-B3PA-2350)

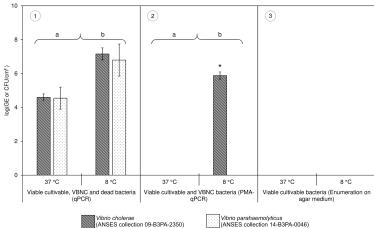


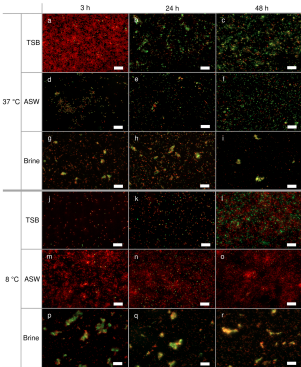
■ Viable cultivable, VBNC and dead bacteria (qPCR)

□ Viable cultivable and VBNC bacteria (PWA-qPCR)

■ Viable cultivable bacteria (Enumeration on agar medium)

Brine, 48 h



Vibrio parahaemolyticus (ANSES collection 14-B3PA-0046)

Vibrio cholerae (ANSES collection 09-B3PA-2350)

