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1 Induction of the Viable but non-culturable state by an alkyl dipropylene 2 triamine-based disinfectant in *Vibrio parahaemolyticus* biofilms

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10 **Abstract:**

11 *Vibrio parahaemolyticus* is a halophilic foodborne pathogen capable of persisting throughout the
12 seafood production chain, from aquaculture farms to processing facilities, by forming biofilms
13 conferring increased tolerance to disinfectants. In this study, the impact of N-(3-aminopropyl)-N-
14 dodecylpropane-1,3-diamine (APDA) was assessed by epifluorescence microscopy on the viability and
15 structure of biofilms formed by 5 strains from a cohort of 14 *V. parahaemolyticus* isolated from seafood
16 products. While with crystal violet staining most strains did not form biofilms on polystyrene
17 microplates, structured biofilms readily formed on stainless steel surfaces, commonly used in seafood
18 processing environments. Treatment with 300 µg·mL⁻¹ of APDA induced changes in biofilm
19 architecture, with the emergence of cell aggregates observable by microscopy. Although a significant
20 reduction in cultivable cells was observed, qPCR and PMA-qPCR showed that viable but non-culturable
21 (VBNC) cells persisted across all strains, raising the risk of bacterial resuscitation. Two strains
22 maintained low levels of cultivable cells post-treatment, suggesting potential tolerance to APDA. These
23 results indicate that although APDA reduces bacterial cultivability, it does not ensure complete biofilm
24 eradication and may promote persistence through structural changes and VBNC induction. This
25 highlights the need for integrated approaches to effectively control *V. parahaemolyticus* in seafood
26 processing environments.

27 **Keyword:** *Vibrio parahaemolyticus*, biofilm, disinfection, VBNC, viability qPCR, food safety

28 **Introduction:**

29 *Vibrio parahaemolyticus* is a Gram-negative bacterium that naturally inhabits coastal marine
30 environments. Its proliferation is closely linked to warmer sea surface temperatures (above 15 °C),
31 making it increasingly prevalent during warmer seasons. As global temperatures rise, temperate
32 regions are becoming new ecological niches for this pathogen¹. Moreover, *V. parahaemolyticus* is a
33 well-known cause of gastroenteritis in humans, often associated with the consumption of seafood.

34 Numerous outbreaks have been documented globally, highlighting its public health relevance. For
35 instance, recent outbreaks between September 2021 and January 2022 involving two strains of *V.*
36 *parahaemolyticus* resulted in 268 infections linked to oyster consumption in Australia². Similarly,
37 between 2019 and 2023, nine outbreaks were reported in Huzhou, China, infecting over 212
38 individuals³. While raw and undercooked seafood remain the primary vectors, they are not the sole
39 sources of contamination. An analysis of outbreaks in Zhejiang, China, from 2010 to 2022 revealed 29
40 incidents involving unexpected food vehicles such as meat, vegetables, beans, eggs, and fruit
41 products⁴. Moreover, 110 outbreaks were linked to cooked seafood, which is typically considered as
42 safe. These findings point to cross-contamination as a significant transmission route. Previous studies
43 have highlighted such cases: one reported the infection of 100 individuals after cooked shrimp became
44 contaminated via contact with infected ice aboard a cruise ship in Spain⁵. Another incident in Korea
45 involved 237 individuals with symptoms of gastroenteritis, including 34 confirmed cases of *V.*
46 *parahaemolyticus* infection. The outbreak was attributed to cross-contamination via contaminated
47 inert surfaces, specifically shared kitchen utensils used between squid and other kimbap ingredients⁶.
48 Cross-contamination from inert surfaces, including food preparation areas and utensils, is often driven
49 by the presence of biofilms. These structured microbial communities are embedded within an
50 extracellular matrix, which confers enhanced resistance to environmental stressors and allows bacteria
51 to persist in otherwise hostile conditions^{7,8}. *V. parahaemolyticus* is known to form biofilms on both
52 living surfaces, such as mussels⁹, prawns, and crab shells¹⁰, as well as on non-living materials like
53 stainless steel¹¹ and polypropylene¹² both of which are commonly found in food processing plants.

54 To prevent the formation of biofilms and the associated risk of cross-contamination, preventive
55 strategies such as routine surface disinfection are critical. Food industry operators rely on a range of
56 disinfectants to control microbial persistence. Among these, quaternary ammonium compounds
57 (QACs) were extensively used, with benzalkonium chloride (BAC) being the most prevalent due to its
58 broad-spectrum antimicrobial activity¹³. However, their utilization has led to the emergence of
59 resistant bacterial strains which possess antimicrobial resistance genes (ARG)^{14,15}. Additionally, recent
60 studies, including Mougín et al.,¹⁶ and Regnier et al.,¹⁷ (Submitted), have shown that exposure of *V.*
61 *parahaemolyticus* biofilms to BAC at concentrations ranging from <25 to 500 µg.mL⁻¹ can induce
62 excessive biofilm production on polystyrene and stainless-steel surfaces, and promote the emergence
63 of VBNC cells. In light of these limitations, alternative disinfectants are being explored, such as those
64 based on alkyldipropylene triamine¹⁸. One of them is the N-(3-aminopropyl)-N-dodecylpropane-1,3-
65 diamine (APDA) which could be used in combination or in alternative to QAC¹⁸. APDA is a cationic
66 surfactant such as BAC and will integrate and disturb the bacteria cell membrane, leading to cell lysis¹⁹.
67 Unlike BAC, the tolerance of *V. parahaemolyticus* biofilms to APDA remains unexplored. To date, only

68 one study has evaluated the effect of APDA on microbial biofilms, specifically phototrophic
69 communities that may include bacteria²⁰. However, no data are currently available regarding its impact
70 on bacterial biofilms, particularly those formed by the emergent human pathogen *V.*
71 *parahaemolyticus*.

72 In this context, the present study aimed to fill this knowledge gap by assessing the impact of APDA on
73 *V. parahaemolyticus* biofilms. Specifically, the efficiency of this treatment on the eradication of
74 biofilms and the impact of this molecule of biofilm populations and morphology were investigated by
75 combining microscopical and molecular approach.

76 **Material and Methods:**

77 ***Vibrio parahaemolyticus* strain collection:**

78 Fourteen *V. parahaemolyticus* strains were isolated between 2016 and 2021 from shrimps and prawns,
79 following the ISO standard 21872-1:2017²¹ (Table 1). Contaminated food samples originated from
80 three countries: Bangladesh (n=1), Ecuador (n=1), Vietnam (n=10), No-determined (n=2). All isolates
81 were cultured on saline nutrient agar (SNA, 2% NaCl; Bio-Rad, Marne-la-Coquette, France) and
82 incubated for 24 hours at 37 °C.

83 **Minimal biofilm eradicable concentration**

84 The minimal biofilm eradicable concentration (MBEC) determination was performed in accordance
85 with the methodology outlined by Mougin et al.,¹⁶. Individual colonies of *V. parahaemolyticus* grown
86 on agar were harvested using a sterile loop and inoculated into 20 mL of Tryptic Soy Broth (TSB)
87 supplemented with 2% (w/v) NaCl (Biokar, Pantin, France; Labkem, Rungis, France). This culture was
88 incubated at 37 °C for 24 hours. Subsequently, a subculture was prepared in fresh TSB 2% NaCl (Tryptic
89 Soy Broth with 2% NaCl), adjusted to approximately 10⁸ CFU/mL using established OD₆₀₀ correlations
90 specific to each strain (data not shown). Aliquots of 200 µL from this suspension were dispensed into
91 wells of a microtiter plate (REF: 353072, Falcon, Waltham, United States). To mitigate evaporation,
92 200 µL of sterile water were added to all edge wells. The plates were then incubated at 22 °C under
93 static conditions for 24 hours to promote biofilm development. As a sterility control, wells containing
94 only TSB 2% NaCl without bacteria were included. Following incubation, biofilms were gently rinsed
95 three times with 200 µL of sterile physiological water (0.85% NaCl) to eliminate planktonic cells.
96 Control biofilm were exposed 20 min to 200µL of sterile physiological water, while treated biofilms
97 were then exposed for 20 minutes to 200 µL of P3-TOPAX 990 (Ecolab, Bagneux, France), a solution
98 containing 4% (w/v) N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (APDA), 4% (w/v) C12–C14
99 Alkyl dimethylamine N-oxides, 1% (w/v) Acetic acid 1% and 0.5% (w/v) Branched and linear C13–C15

100 ethoxylated alcohols. The disinfectant solution was diluted in sterile water to achieve final APDA
101 concentrations of 24, 52, 100, 200, 300 and 400 $\mu\text{g}\cdot\text{mL}^{-1}$. After treatment, the disinfectant was removed
102 and the wells were incubated with 200 μL of NP2X neutralizer (Actalia, Saint-Lô, France) for 30 minutes.
103 The neutralizer was then discarded, and the biofilms were rinsed three more times with sterile
104 physiological water. To assess the residual biomass, the wells were stained with 200 μL of 0.1% (v/v)
105 crystal violet (CV; Condalab, Madrid, Spain) for 20 minutes at room temperature, followed by three
106 washes with sterile water. The plates were air-dried for approximately 2 minutes. Subsequently,
107 200 μL of 70% ethanol were added to each well to solubilize the bound dye, gently pipetting to
108 facilitate elution. Optical density at 595 nm (OD_{595}) was then measured using a SAFAS Xenius XC
109 spectrofluorometer (SAFAS, Monaco, France). Each measurement was performed in biological
110 triplicate, with blank controls. Final OD_{595} readings were adjusted by subtracting the mean blank
111 values; any resulting negative values were recorded as zero.

112 **Disinfection of stainless coupons**

113 Rectangular coupons (40 mm \times 20 mm) of AISI 316 L 2B stainless steel (Sapim Inox, Loison-sous-Lens,
114 France) were prepared by cleaning and sterilizing them according to the procedure described by
115 Brauge et al.,²². After sterilization, the coupons were placed in Petri dishes and left to dry overnight at
116 room temperature within a microbiological safety cabinet. For subsequent use, two coupons were
117 arranged in a 90 mm Petri dish (Greiner, Frickenhausen, Germany), which was then set inside a larger
118 145 mm Petri dish (Greiner) containing a paper towel moistened with sterile water to maintain
119 humidity.

120 **Biofilm formation and APDA treatment on stainless steel coupons**

121 Biofilms were cultivated and processed largely following the method of Brauge et al.,²³, with slight
122 adjustments. Isolated colonies of *V. parahaemolyticus* were picked from SNA agar plates (Bio-Rad)
123 using a sterile loop and inoculated into 20 mL of TSB 2% NaCl, then incubated overnight at 37 °C. The
124 overnight cultures were subsequently standardized to approximately 10^8 CFU/mL in fresh TSB 2% NaCl.
125 A volume of 20 mL from this adjusted suspension was dispensed into 90 mm Petri dishes (Nunc,
126 Roskilde, Denmark), each containing a previously cleaned and sterilized stainless-steel coupon. These
127 setups were incubated at 22 °C for 24 hours to facilitate biofilm development. After incubation, loosely
128 attached bacteria were removed by rinsing. The coupons placed in a 90 mm Petri dish were immersed
129 three consecutive times in 12.5 mL of sterile physiological water (0.85% NaCl), ensuring complete
130 submersion during each rinse.

131 Biofilms were then exposed to treatment by immersing the coupons, in the same way, previously
132 described, in 12.5 mL of sterile physiological water for control or in a P3-TOPAX 990 solution prepared

133 to achieve 300 $\mu\text{g}\cdot\text{mL}^{-1}$ APDA, for 20 minutes at room temperature. After treatment, the disinfectant
134 or water was discarded, and the coupons were transferred into 12.5 mL of NP2X disinfectant
135 neutralizer, where they remained for 30 minutes at room temperature. Finally, the coupons were
136 rinsed three additional times by immersion in sterile physiological water to complete the procedure.

137 **Biofilm observations by epifluorescence and scanning electron microscopy**

138 After treatment with either sterile water (serving as the control) or APDA (300 $\mu\text{g}\cdot\text{mL}^{-1}$), the liquid was
139 removed, and the stainless-steel coupons were immersed in 12.5 mL of NP2X disinfectant neutralizer
140 (Actalia, Saint-Lô, France) for 30 minutes at room temperature. This step was followed by three rinses,
141 each involving immersion in 12.5 mL of sterile physiological water. Next, 5 *V. parahaemolyticus*
142 biofilms were subjected to staining procedures to evaluate cell viability and to visualize specific
143 components of the extracellular matrix. Cell viability within the biofilms was assessed using the
144 LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, Thermo Fisher Scientific) according to the
145 manufacturer's guidelines. To stain extracellular DNA visualization, biofilms were treated with 1 μM
146 Dito™-1 (Bioquest) for 30 minutes in the dark, followed by a rinse with sterile physiological water.
147 Fluorescence imaging of the biofilms was carried out using an Imager.Z1 epifluorescence microscope
148 (Zeiss, Oberkochen, Germany) equipped with an Axiocam MRm CCD camera (Zeiss), employing filter
149 sets appropriate for each fluorophore. For every staining condition, a minimum of three representative
150 fields of view were captured to document the biofilm structures.

151 Additionally, the architecture of the biofilms was examined by scanning electron microscopy (SEM;
152 Jeol, SM-IT210, Japan), following the procedure detailed by Brauge et al.,²².

153 **Quantification of total and viable (culturable and non-culturable) biofilm populations**

154 Biofilms were collected by vigorously swabbing the surfaces of the stainless-steel coupons with a
155 sterile dry cotton swab (Deltlab, Barcelona, Spain), which was then immersed in an Eppendorf tube
156 containing 2.5 mL of TSB 2% NaCl. To enhance biofilm recovery, the coupon was swabbed a second
157 time using the same swab. This process was then repeated with a new swab. Both swabs were
158 subsequently broken off into the same tube and vortexed for 20 sec to thoroughly disperse the biofilm
159 cells into suspension. This homogenized suspension served as the source for quantifying total and
160 viable cell populations, encompassing both viable culturable (VC) and VBNC cells.

161 Total and viable populations were assessed using a slightly modified version of the protocol established
162 by Brauge et al.,²³. For assessing viable cells specifically, an aliquot of 495 μL of each sample was
163 treated with propidium monoazide (PMA; $\text{C}_{21}\text{H}_{33}\text{Cl}_2\text{N}_6$; Biotium, Hayward, USA) at a final concentration
164 of 50 μM . Following incubation in the dark, samples were exposed to intense light (100% intensity) for

165 10 minutes using a PhAST Blue lamp (GenIUL, Barcelona, Spain) while remaining in their tubes.
166 Concerning quantification of total population, an aliquot of 495 μL were made and no PMA-treatment
167 were realized. After those steps, all samples were centrifuged at $5,000 \times g$ for 10 minutes, and DNA
168 was extracted using the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's protocol for
169 "Purification of Total DNA from Animal Tissues," utilizing ATL buffer for lysis. Relative DNA
170 quantification targeted the *toxR* gene, which encodes a membrane-associated regulatory protein.
171 Quantitative PCR (qPCR) was carried out using qPCR kit LightCycler[®] 480 Probs Master (Roche, Basel,
172 Switzerland) in 20 μL reactions containing 7.24 μL PCR-grade water, 10 μL of Roche Master Mix 2X, 900
173 nM each of forward (5'- GAA CCA GAA GCG CCA GTA GT -3') and reverse (5'- AAA CAG CAG TAC GCA
174 AAT CG -3') primers specific to *V. parahaemolyticus* (Eurogentec, Seraing, Belgium), 200 nM of a
175 FAM/TAMRA-labeled *toxR* probe (5'-TCA CAG CAG AAG CCA CAG GTG C-3') (Eurogentec), and 2 μL of
176 extracted DNA. Amplification was performed on a LightCycler 480 system (Roche) under the following
177 cycling conditions: an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 15 seconds
178 at 95 °C and 90 seconds at 60 °C. Each qPCR run plate included: (i) one DNA sample treated with PMA,
179 with and without ADPA treatment; (ii) one DNA sample untreated with PMA, with or without ADPA
180 treatment; (iii) a standard curve well; (iv) a reaction mix control well; and (v) a negative control well.

181 Genome equivalents (GE) were used to quantify both total and viable populations, calculated using the
182 *V. parahaemolyticus* genome size of 5.1×10^6 bp determined by Witherall et al.,²⁴. Three standard
183 curves (Supplementary Figure 1) were generated during this study and refreshed every 14 days in order
184 to prevent DNA degradation. The standard curve spanning from 10^9 GE/mL to 5×10^1 GE/mL and
185 prepared using *V. parahaemolyticus* strain 14B3PA0046. *Vibrio alginolyticus* CIP 71.3 genomic DNA
186 adjusted to $50 \mu\text{g} \cdot \text{mL}^{-1}$ served as a negative control.

187 For determining culturable populations, a 1 mL fraction from the control sample was serially diluted
188 up to 10^{-4} . Meanwhile, undiluted APDA-treated samples and 10^{-2} and 10^{-4} dilutions of control samples
189 were plated on SNA agar using spiral plating easySpiral Pro (Interscience, Saint Nom la Bretèche,
190 France). After incubating plates for 24 hours at 37 °C, colonies were counted using an automated
191 colony counter (Scan 500[®], Interscience, Saint Nom la Bretèche, France). The discrepancy between the
192 total and viable population counts was interpreted as the proportion of dead cells. Likewise, the
193 difference between the viable and culturable populations was used to infer the presence of VBNC cells.

194 **Statistical analysis**

195 Statistical analyses were conducted using RStudio version 2023.12.1+402. Welch's two-sample t-test
196 was applied ($\alpha = 0.05$), considering results significant when the *p-value* was ≤ 0.05 . Data visualization

197 related to population quantification was carried out with Excel version 2108 (Microsoft, Redmond,
198 United-State).

199 **Results**

200 **Determination of the minimal concentration of APDA to eradicate *V. parahaemolyticus* biofilms**

201 To assess the impact of the alkyl dipropylene triamine compound APDA, the MBEC for 14 *V.*
202 *parahaemolyticus* strains was determined (Figure 1). In the absence of treatment (0 $\mu\text{g}\cdot\text{mL}^{-1}$ APDA), 13
203 strains did not form detectable biofilms ($\text{OD}_{595} = 0$). Only strain 17-B3PA-1510 showed a different
204 biofilm-forming ability, with an OD_{595} of 0.3. When treated with increasing concentrations of APDA
205 (ranging from 24 to 400 $\mu\text{g}\cdot\text{mL}^{-1}$), no significant effect was observed for most strains. However, strain
206 17-B3PA-1510 exhibited an increase of OD_{595} reaching 0.97 after treatment.

207 **Matrix component and cell density of *V. parahaemolyticus* biofilms observed by epifluorescence and** 208 **scanning electron microscopy**

209 The impact of a 300 $\mu\text{g}\cdot\text{mL}^{-1}$ APDA treatment was assessed by microscopy using live/dead cell staining
210 and labelling two biofilm extracellular matrix component: extracellular DNA and polysaccharides
211 (Figure 2). After water treatment, strain 19-B3PA-0924 formed a uniform cells distribution biofilm,
212 composed of viable cells (green) and dead cells (red) (Figure 2A). APDA treatment altered the biofilm
213 architecture, resulting in the formation of cells aggregate. These treated biofilms were predominantly
214 composed of dead cells (red) with a few remaining viable cells (green). Dito™-1 staining revealed a
215 well-developed extracellular matrix in untreated biofilms, confirming their uniform cells distribution
216 (Figure 2B). In APDA-treated biofilms, microscopy confirmed the presence of large cells aggregate rich
217 in extracellular DNA (Figure 2C). Similar results were obtained for the biofilms formed by strains 18-
218 B3PA-0236, 17-B3PA-1510, 21-B3PA-1227, and 19-B3PA-2027 (Supplementary Figure 2).

219 Scanning electron microscopy (SEM) analyses further confirmed the presence of cells aggregate in
220 APDA-treated biofilms for strain 19-B3PA-0924 (Figure 3B), in contrast to the uniform cells distribution
221 architecture with isolated cells observed in water-treated controls (Figure 3A). Bacterial cells in treated
222 biofilms also appeared damaged (Figure 3D), whereas those in untreated biofilms retained their
223 characteristic bacillary morphology (Figure 3C).

224 **Quantification of *Vibrio parahaemolyticus* populations according to their viability and culturable** 225 **status in biofilms treated with APDA**

226 Total bacterial populations (viable and dead) and viable populations (including culturable and non-
227 culturable cells), were quantified in the biofilms formed by the 14 strains, both without treatment
228 (water control) and with APDA treatment (Figure 4).

229 Following the water treatment, the total bacterial load in the biofilms ranged from 6.14 Log GE/cm²
230 for strain 19-B3PA-2683 to 8.50 Log GE/cm² for strain 21-B3PA-1227. According to qPCR and PMA-
231 qPCR analyses, no dead cells were detected among the 14 tested strains. However, dead populations
232 were still visible by microscopy (Figure 2), suggesting that their abundance was too low to be quantified
233 by molecular methods. Culturable populations (VC) were detected in all biofilms, ranging from 3.60
234 Log CFU/cm² for strain 19-B3PA-0924 to 6.74 Log CFU/cm² for strain 21-B3PA-1227. The differences
235 between viable and culturable populations revealed the presence of VBNC cells in all untreated
236 biofilms. The extent of the VBNC fraction was particularly high (greater than 2.67 Δ Log, corresponding
237 to 463 fold) in biofilms formed by strains 16-B3PA-2613, 17-B3PA-3962, 19-B3PA-0924, 18-B3PA-1732,
238 18-B3PA-0236, 17-B3PA-1510, and 21-B3PA-0171. In the remaining strains, the magnitude of the VBNC
239 population ranged from 0.75 Δ Log (6 fold; strain 21-B3PA-1227, the lowest value) to 1.54 Δ Log (35
240 fold; strain 18-B3PA-2680).

241 After treatment with 300 μ g·mL⁻¹ of APDA, no culturable population were quantified in all biofilms,
242 except for strain 17-B3PA-1512, where residual populations of 1.25 Log CFU/cm² were detected. For
243 strain 21-1227, residual cells were occasionally observed, but the mean values were below the
244 detection limit and therefore considered negative. This sharp reduction in culturable cells was
245 accompanied by a significant increase in the VBNC fraction across all biofilms, with the highest value
246 observed in strain 21-B3PA-0171 (7.20 Δ Log, corresponding to a 1.57×10^7 fold difference). These
247 increases were statistically significant as confirmed by Welch's two-sample t-test ($\alpha = 0.05$), with P
248 values ranging from 3.56×10^{-2} for strain 17-B3PA-0924 to 1.95×10^{-6} for strain 18-B3PA-0236. A dead-
249 cell population was also detected in all treated biofilms, except for strains 18-B3PA-1732, 21-B3PA-
250 0171, and 21-B3PA-1227. In contrast, total and viable populations remained largely unaffected by the
251 treatment, with the exception of strain 19-B3PA-2027, which showed a significant decrease in its viable
252 population ($P = 2.28 \times 10^{-2}$; $\alpha = 0.05$).

253 **Discussion**

254 This study aimed to evaluate the impact of APDA on *V. parahaemolyticus* biofilms, specifically
255 investigating whether specifically investigating whether treatment effectively eradicates biofilms and
256 whether this molecule has an impact on biofilm populations and morphology. MBEC assays were
257 performed on 14 *V. parahaemolyticus* strains isolated from seafood products. To our knowledge, this
258 is the first study to evaluate the effect of an alkyl dipropylene triamine compound on *V.*

259 *parahaemolyticus* biofilms. Under these experimental conditions, 13 out of the 14 strains failed to form
260 detectable biofilms on polystyrene microplates, which contrast with findings of Mougin et al.,¹⁶
261 obtained using different strains. Due to the absence of biofilm formation, no MBEC values could be
262 determined. However, for strain 17-B3PA-1510, the only strain capable to form biofilm under these
263 conditions, biomass increased following treatment with 100 and 200 $\mu\text{g.mL}^{-1}$ of APDA. This
264 phenomenon of biofilm overproduction in response to disinfectant exposure has already been
265 described for *V. parahaemolyticus* biofilms treated with benzalkonium chloride (BAC)¹⁷. In a previous
266 study, we demonstrated that strains lacking the *qacEΔ1* gene (which encodes a SMR-family efflux
267 pump conferring quaternary ammonium (QACs) tolerance) overproduced biofilm matrix when
268 exposed to BAC¹⁷. The proposed hypothesis is that, in the absence of resistance determinant, bacteria
269 trigger an adaptive stress response based on matrix overproduction to limit disinfectant diffusion.
270 Conversely, strains carrying *qacEΔ1* rely more on active efflux of BAC, which reduces the need for
271 matrix production but may impose energetic cost limiting biomass accumulation. For strain 17-B3PA-
272 1510, whole-genome sequencing confirmed the absence of the *qacEΔ1* gene (accession number:
273 SAMN48767444). This strain nevertheless harboured several efflux pump genes belonging to the
274 Resistance-Nodulation-Division (RND) family with a complete set of *vme* and *vpo* genes (*vmeA*, *vmeB*,
275 *vmeC*, *vmeD*, *vmeE*, *vmeF*, *vmeG*, *vmeH*, *vmeI*, *vmeJ*, *vmeK*, *vmeP*, *vmeQ*, *vmeT*, *vmeU*, *vmeV*, *vmeY*,
276 *vmeZ*, and *vpoC*) associated with the RND system and its outer membrane components^{25,26}, as well as
277 *vmrA*, from the Multidrug and Toxic Compound Extrusion (MATE) system. However, since these efflux
278 genes were also present in all other tested strains, they cannot alone explain the specific phenotype
279 observed in strain 17-B3PA-1510. Another hypothesis is that exposure of strain 17-B3PA-1510 to the
280 disinfectant specifically stimulated the production of components inducing directly or indirectly biofilm
281 formation in *V. parahaemolyticus*. Two non-exclusive hypotheses may explain the observed increase
282 in biofilm biomass. The first is supported by the work of Mougin et al. (2024)¹⁶, who demonstrated that
283 BAC treatment could, depending on the strain, either induce or not an increase in c-di-GMP, a second
284 messenger known to stimulate biofilm formation and matrix overproduction. Indeed, our observations
285 with strain 17-B3PA-1510 suggest that short-term exposure to disinfectants may act as a rapid stress
286 signal, triggering c-di-GMP-dependent regulatory pathways and promoting matrix production within
287 minutes. Such a mechanism is consistent with previous reports showing that c-di-GMP levels can
288 rapidly increase in response to environmental signals in other bacterial species, such as *Pseudomonas*
289 *aeruginosa*²⁷ or *Listeria monocytogenes*²⁸. The second hypothesis is that disinfectant exposure altered
290 the biofilm architecture by promoting the formation of aggregates, as revealed by our SEM
291 observations. These structural changes could increase the retention of crystal violet, thereby
292 contributing to the apparent rise in measured biomass.

293 To better reflect the conditions encountered throughout the seafood industry, *V. parahaemolyticus*
294 biofilms were cultivated and treated on stainless steel, a material widely used across the seafood and
295 aquaculture processing chain²⁹. The impact of APDA was assessed through microscopic observations
296 combined with live/dead staining to visualize bacterial populations based on their viability status, as
297 well as specific staining targeting key components of the extra cellular matrix: extra cellular DNA and
298 exo-polysaccharides. Our results showed that, unlike what was observed on polystyrene, *V.*
299 *parahaemolyticus* was able to form biofilms on stainless steel. These findings are consistent with
300 several studies reporting that the nature of the surface, particularly its hydrophilicity, roughness, or
301 charge can influence bacterial adhesion and biofilm formation in marine bacteria, including *V.*
302 *parahaemolyticus*^{30,31}. Indeed, a study of Mizan et al.,³² highlighted that *V. parahaemolyticus* had a
303 greater variation in biofilm formation in microtiter plate (hydrophobic surface) than on stainless steel
304 (hydrophilic surface), and that it may vary depending on the strain and experimental conditions.

305 Microscopic observations further revealed that treatment with 300 µg.mL⁻¹ of APDA did not fully
306 eradicate *V. parahaemolyticus* biofilms formed on stainless steel. The remaining biofilms were mainly
307 composed of dead cells (red staining), but also included a few viable cells (green staining), along with
308 an extracellular matrix primarily composed of extracellular DNA.

309 To pursue our investigation, the total bacterial populations and viable populations (including both
310 culturable and non-culturable cells) in the biofilms formed by the different *V. parahaemolyticus* strains
311 were quantified. This was done using enumeration on agar media, qPCR and PMA-qPCR. These
312 methods also allowed to estimate the proportion of VBNC (Viable But Non-Culturable) and dead cells
313 under each condition. It is important to note certain methodological limitations. As with any nucleic
314 acid extraction method, partial DNA loss may occur during the process, which could lead to an
315 underestimation of the total amount of DNA. Such technical constraints may influence the
316 quantification of total and viable populations by qPCR and PMA-qPCR, respectively. In our study, this
317 potential bias affected control and APDA-treated samples in a similar way, thereby allowing a reliable
318 comparison between the two conditions. The results confirmed that following disinfectant treatment,
319 VBNC populations were present in the biofilms of all 14 strains tested. Although no data are currently
320 available on the effect of APDA on the viability of *V. parahaemolyticus* biofilms, these findings are
321 consistent with previous observations using other disinfectants. For example, Mougín et al.¹⁶ and
322 Régnier et al.¹⁷ (Submitted) demonstrated, respectively using flow cytometry and PMA-qPCR, the
323 persistence of *V. parahaemolyticus* in a VBNC state after BAC treatment. It has also been shown that
324 *V. parahaemolyticus* can enter a VBNC state in response to stress caused by more common cleaning
325 agents like sodium hypochlorite and resuscitate after treatment. Indeed Wang et al.³³ showed that

326 planktonic *V. parahaemolyticus* cells treated with 40 mg/L of chlorine enter in VBNC state and could
327 recover viability after 24 hours. In the present study, the observed recovery time was around 7 days
328 (data not shown), which is longer, and this difference may be due to variations in the resuscitation
329 protocol, such as the use of sodium hypochlorite instead of APDA, and the omission in the present
330 study of catalase and sodium pyruvate, which are known to enhance recovery³⁴. Similarly, a study by
331 Osland et al.³⁵ showed that 48-hour-old *E. coli* biofilms exhibited a loss of cultivability, with an average
332 log reduction of 0.95 after 15 minutes and 1.08 after 30 minutes of treatment with APDA. In contrast,
333 here, a much higher reduction of approximately 6.34 Log (GE/cm²) was observed. It may be due *E. coli*
334 intraspecies biofilms properties which confer higher tolerance to APDA than *V. parahaemolyticus*.
335 Interestingly, VC populations were detected after treatment with 300 µg.mL⁻¹ of APDA in one strains:
336 17-B3PA-1511. The possible presence of previously described resistance genes in this strain may
337 explain the persistence of a culturable population after APDA treatment but no direct link has been
338 determined. *Some apparently contradictory results were observed when comparing live/dead*
339 *microscopy observations with molecular quantification data.* In particular, red-stained populations
340 (dead cells) were observed by microscopy but could not be quantified by molecular methods. This
341 phenomenon was especially evident when *V. parahaemolyticus* biofilms were treated with water. It is
342 therefore important to emphasize certain methodological limitations regarding the interpretation of
343 results obtained from microscopy and molecular biology approaches. Live/dead microscopy should
344 primarily be considered a qualitative tool: propidium iodide can penetrate stressed but still viable cells,
345 or bind to extracellular DNA, leading to an overestimation of “dead” cells^{36,37}. In contrast, PMA-qPCR
346 provides a quantitative measurement at the scale of the entire sample, but may underestimate dead
347 cells if dye penetration is limited in dense biofilms or if DNA from lysed cells is poorly recovered during
348 extraction³⁸⁻⁴⁰. These methodological differences explain why microscopy revealed more red-stained
349 cells than PMA-qPCR. Nevertheless, both approaches converge on a key finding: the persistence of
350 viable populations after BAC exposure, confirming the induction of a VBNC state.

351 Eventually, treatment with APDA altered the morphology of *V. parahaemolyticus* biofilms, leading to
352 the formation of large cells aggregate. A study by Mougín et al.¹⁶ demonstrated that treatment with
353 1.25% benzalkonium chloride for 24 hours altered the architecture of *V. parahaemolyticus* biofilms by
354 enhancing the presence of cells aggregates. The authors hypothesized that these architectural
355 differences may constitute a reinforced physical barrier, limiting the diffusion of antimicrobial
356 compounds. Therefore, the emergence of cell aggregates may partly explain why, in all biofilms of the
357 present study, APDA treatment resulted in a complete loss of culturability rather than cell death.
358 Similar hypotheses have been proposed in other studies, particularly in the context of *Listeria*
359 *monocytogenes*. For instance, it has been suggested that *L. monocytogenes* mutants exposed to

360 alkaline treatments modified their biofilm architecture in a way that promotes denser structures,
361 thereby reducing disinfectant efficacy⁸. Study of Kocot et al.,⁴¹ showed that dual species biofilm
362 (*Staphylococcus aureus* or *Pseudomonas aeruginosa* with *Listeria innocua*) formed aggregates after
363 exposition to quaternary ammonium or tertiary alkyl amine based disinfectant.

364 **Conclusion**

365 This study aimed to evaluate the impact of N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (APDA)
366 on *V. parahaemolyticus* biofilms, specifically investigating whether treatment effectively eradicates
367 biofilms and whether this molecule has an impact on biofilm populations and morphology. While MBEC
368 tests showed that most strains did not form substantial biofilms on polystyrene under the tested
369 conditions, some biomass increases were observed following APDA exposure in one strain. However,
370 when biofilms were grown on stainless steel, a material more representative of seafood processing
371 environments *V. parahaemolyticus* developed structured biofilms that were not fully eradicated by
372 APDA treatment at 300 µg.mL⁻¹. Microscopic analyses revealed significant changes in biofilm
373 architecture after treatment, including the appearance of dense cells aggregates potentially
374 contributing to antimicrobial tolerance. Importantly, although the treatment drastically reduced
375 cultivable populations, VBNC cells persisted in all tested strains, representing a major concern for food
376 safety. In two strains, residual cultivable cells were still detected after treatment, indicating a potential
377 resistance to APDA. These findings highlight the need for further investigation into the behaviour of *V.*
378 *parahaemolyticus* biofilms under realistic conditions and stress and the importance of evaluating
379 disinfection strategies not only for their ability to reduce bacterial counts on agar media but also for
380 their effects on microbial persistence and viability.

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490 **Authors contribution statement**

491 A.R Writing – original draft, Conceptualization Ideas, Investigation, Visualization; E.C Writing – review
492 & editing, Formal analysis; C.D Writing – review & editing, Formal analysis; G.M Writing – review &
493 editing, Funding acquisition, Project administration; M.G Writing – review & editing, Project
494 administration, Supervision; T.B Writing – review & editing, Conceptualization Ideas, Funding
495 acquisition, Project administration, Supervision.

496 **Competing interests**

497 The author(s) declare no competing interests.

498 **Data Availability Statement**

499 The datasets generated during and/or analysed during the current study are available from the
500 corresponding author on reasonable request.

501 **Funding Declaration**

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503 **Figure caption**

504 Figure 1: Minimum Biofilm Eradication Concentration (MBEC) of *Vibrio parahaemolyticus* treated
505 with N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (APDA) (n=14). OD(595): Optical density at
506 595nm.

507 Figure 2: Epifluorescence microscopy of *Vibrio parahaemolyticus* biofilms formed on stainless steel at
508 22 °C for 24 h and treated with either sterile water (control) or 300 µg.mL⁻¹ N-(3-aminopropyl)-N-
509 dodecylpropane-1,3-diamine (APDA treated). Biofilms from strain 19-B3PA-0924 were stained to
510 assess different biofilm components: (A) LIVE/DEAD® staining showing viable cells, (B) LIVE/DEAD®
511 staining showing dead cells, (C) LIVE/DEAD® staining showing merged viable and cells, (D) Dito™-1
512 staining for extra-cellular DNA,

513 Figure 3: Scanning electron microscopy of *Vibrio parahaemolyticus* biofilms of strain 19-B3PA-0924
514 formed on stainless steel at 22 °C for 24 h and treated with either sterile water (Water control) or
515 300 µg.mL⁻¹ N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (APDA treated). (A) Biofilm treated
516 with water, (B) Biofilm treated with APDA, (C) Cells treated with water, (D) Cells treated with APDA.

517 Figure 4: Impact of 300 µg.mL⁻¹ N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (APDA) treatment
518 on *Vibrio parahaemolyticus* biofilms population. Quantification of total, viable, and culturable
519 populations in biofilms formed. * Indicates statistically significant differences (Welch's two sample t-
520 test; p < 0.05; α = 0.05) between the water control and APDA treated biofilms. All strains ID were
521 simplified removing B3PA for more readability. The limit of detection were set at 1.3 Log(CFU/cm²).

522

523