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## Monitoring the freshness of fish: development of a qPCR method applied to MAP chilled whiting

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1 **Monitoring the freshness of fish: development of a qPCR method applied to MAP chilled**  
2 **whiting**

3

4 Running title : qPCR method targeting *torA* applied to monitoring the freshness of fish

5

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## ABBREVIATIONS

bp: Base pair, Cq: Cycle quantification, HyC: Hybridisation coefficient, IAC: Internal amplification control, MAP: Modified atmosphere packed, SSO: Specific spoilage organisms, TMA: Trimethylamine, TMAO: Trimethylamine N-oxide, TSA-YE: Tryptone soya agar - yeast extract, TVB-N: Total volatile basic nitrogen, TVC: Total viable count.

## ABSTRACT

**BACKGROUND:** Monitoring of early stages of freshness decay is a major issue for the fishery industry to guarantee the best quality for this highly perishable food matrix. Numerous techniques have been developed, but most of them have the disadvantage of being reliable only either in the last stages of fish freshness or for the analysis of whole fish. This study describes the development of a qPCR method targeting the *torA* gene harboured by fish spoilage microorganisms. *torA* encodes an enzyme that leads to the production of trimethylamine responsible for the characteristic spoiled-fish odour.

**RESULTS:** A degenerate primer pair was designed. It amplified *torA* gene of both *Vibrio* and *Photobacterium* with good efficiencies on 7-log DNA dilutions. The primer pair was used during a shelf-life monitoring study achieved on modified atmosphere packed, chilled, whiting (*Merlangius merlangus*) fillets. The qPCR approach allows detecting an increase of *torA* copies throughout the storage of fillets in correlation with the evolution of both total volatile basic nitrogen (-0.86) and trimethylamine concentrations (-0.81), known as spoilage markers.

**CONCLUSION:** This study described a very promising, sensitive, reliable, time-effective, technique in the field of freshness characterisation of processed fish.

**Keywords:** *torA*, qPCR, modified atmosphere packed fish , freshness assessment.

## INTRODUCTION

45

46 The fishing industry is a sector in constant evolution which in Europe has been committed to  
47 quality assurance processes for nearly twenty years, <sup>1,2</sup> in response to the expectations of both  
48 consumers and the professionals in this sector. Quality assurance in the food and feed industry is a  
49 concept that includes many factors such as food safety, freshness, nutritional composition, and  
50 organoleptic properties; these can be affected by numerous parameters along the supply chain,  
51 such as catching conditions, handling, processing and storage. <sup>3</sup> The major issue is thus to develop  
52 tools to improve quality control through traceability, good production practices and laboratory  
53 analysis.

54 The fish freshness state could be defined as the early phase of shelf-life. In fishery products, there  
55 is a particularly close relationship between freshness and quality. Indeed, numerous studies using  
56 quality indicators established that the global quality of fish decreases with the increase of shelf-  
57 life, <sup>4-6</sup> while a development of spoilage and/or pathogenic flora is recorded. <sup>7</sup> Fish spoilage is the  
58 result of the variation of physical, biochemical and microbial parameters in fish muscle. Spoilage  
59 flora is one of the major causes of quality decay in fish products, responsible for the alteration of  
60 organoleptic properties <sup>7,8</sup> and the production of hazardous molecules such as biogenic amines. <sup>9</sup>

61 Generally, fish quality can be assessed using a wide range of techniques such as sensory, chemical  
62 or microbiological analyses. <sup>10-13</sup>

63 For a long time, fish quality was assessed in the fish industry through sensory analysis by well-  
64 trained in-house panel, with the evaluation of many parameters characterizing skin, eyes, gills or  
65 texture thanks to methods as Torry scheme, EU scheme or more recently QIM. <sup>1,10</sup> Unfortunately,  
66 much skills were lost with the arrival of new staff, very few of whom were experienced in fish  
67 quality assessment. <sup>14</sup> Despite this problem, sensory analysis remains one of the most suitable  
68 methods of assessing fish freshness; although it suffers from drawbacks such as panel subjectivity  
69 and training costs or the impact of environmental factors. <sup>10</sup>

70 Chemical analysis can be routinely used to assess the freshness of fish, using total volatile basic  
71 nitrogen analysis (TVB-N). This technique is particularly suitable for species belonging to the  
72 *Pleuronectidae*, *Merlucciidae* and *Gadidae* families and redfish (*Sebastes* spp.), but it is more a  
73 rejection tool than a monitoring method. Indeed, TVB-N analysis is usually described as  
74 unsuitable during the first 10 days of chilled storage.<sup>15</sup> Rejection values have been fixed for three  
75 different groups of species. For the group comprising *Merlucciidae*, *Gadidae* and Atlantic salmon  
76 (*Salmo salar*) the maximum TVB-N value accepted has been set to 35 mg of nitrogen/100 g.<sup>16</sup>  
77 The major drawback concerning TVB-N analysis is that there is a large range of fish, commonly  
78 classified as oily fish such as *Scombridae*, *Clupeidae* or sea bass (*Dicentrarchus labrax*),<sup>17</sup> for  
79 which it cannot be used for assessing freshness nor can it be used for numerous kinds of processed  
80 fish.

81 The microbiology of fish is well documented<sup>7, 18, 19</sup> and the microbial ecology of the entire flora  
82 observed on fish has been described.<sup>20, 21</sup> In addition, two concepts have been defined: spoilage  
83 flora, describing all species able to spoil a food matrix, and specific spoilage organisms (SSOs).  
84 SSOs are a restricted range of the spoilage flora, active depending on the conservation process,  
85 such as *Shewanella putrefaciens* on chilled marine fish and *Photobacterium phosphoreum* on CO<sub>2</sub>  
86 and modified atmosphere packed (MAP) chilled fish.<sup>18</sup> A selective and sensitive conductance  
87 method exists allowing quantification of *Photobacterium* between 15 and 55 hours,<sup>22</sup> but as  
88 numerous microbiological methods, the major drawback of such technique is that they are highly  
89 time-consuming. Despite this knowledge, to date no microbial techniques are routinely used to  
90 assess fish freshness. Nonetheless, one of the metabolites synthesised by SSOs, trimethylamine  
91 (TMA), contributes to the characteristic odour of spoiled fish and can be considered as a  
92 fingerprint of the presence of SSOs. Most TMA production is the result of trimethylamine N-oxide  
93 (TMAO) reductase (EC 1.6.6.9) activity<sup>23</sup> encoded by the *torA* gene part of *tor* operon.<sup>24</sup> This  
94 gene is documented in the literature<sup>25, 26</sup> with numerous sequences available in databases such as  
95 GenBank and EMBL thanks to whole genome sequencing of various *Vibrio* strains.

96 Thus the aim of this study was to explore a new field of investigation for fish freshness assessment  
97 that had only been slightly explored so far.<sup>27,28</sup> Based on a previous study,<sup>27</sup> a new qPCR method  
98 was developed targeting the *torA* gene, a marker of specific spoilage flora. This study first aimed  
99 to design new primers with a reduced number of degenerate nucleotides using a new design  
100 strategy. Particular care was paid to decreasing the amplicon size, providing more insight into the  
101 specificity and efficiency of the developed primer pair and amplifying the *torA* gene in SSOs other  
102 than *S. putrefaciens*. The developed technique was applied, along with other routine freshness  
103 estimation methods, to a shelf-life study on MAP whiting (*Merlangius merlangus*) fillets. Whiting  
104 was chosen as it was the fourth most consumed species in France in 2012 and because MAP fish is  
105 increasingly popular among fish consumers.

## 106 MATERIAL AND METHODS

### 107 Bacterial strains and culture conditions

108 Numerous strains were used to carry out the development of this qPCR method. Five strains from  
109 the genera *Shewanella*, *Photobacterium* and *Vibrio*, preferentially originating from sea water or  
110 sea products, were used for the optimisation of the quantitative PCR (Table 1). Additional strains  
111 were used for a specificity study, consisting of *Vibrio* strains from the Centre de Ressources  
112 Biologiques de l'Institut Pasteur (Paris, France) and strains from the American Type Culture  
113 Collection (ATCC) (Manassas, VA, United States) (Table 1). Finally, ten strains from the  
114 laboratory's own collection were also added to this specificity study (Table 1). Four of these  
115 strains were previously isolated from a spoiled whiting (*M. merlangus*) and identified as  
116 *Shewanella baltica* thanks to 16S rRNA and *gyrB* gene sequencing;<sup>29</sup> the remaining six were  
117 previously isolated from different fish industry environments and were identified by 16S rRNA  
118 gene sequencing.

119 All strains were grown in aerobic conditions. All *Vibrio* strains were grown on tryptone soya agar -  
120 yeast extract (TSA-YE) plates using CM0131 base from Oxoid (Dardilly, France) complemented  
121 with NaCl 1.5% (w/v) at 30 °C for 24 h. All *Photobacterium* and *Shewanella* strains were grown

122 under the same conditions but the incubation temperature was lowered to 25 °C. The six strains  
123 from the fish industry were incubated on TSA-YE plates at 30 °C for 24 h. Strains purchased at  
124 ATCC were grown on TSA-YE plates at 37 °C for 24 h, except *E. coli* which was grown at 44 °C  
125 for 24 h and *S. enterica* grown on nutritive agar from Biorad (Marnes-la-Coquette, France).

#### 126 **DNA extraction from pure culture**

127 Firstly, a control of the homogeneity of CFU on plates, for instance shape, texture and colour, and  
128 Gram colorations were performed as basic controls of purity. Then, a loop of biomass was  
129 harvested on plates and subjected to DNA extraction with the DNeasy Blood & Tissue Kit from  
130 Qiagen (Courtaboeuf, France). The manufacturer's instructions were then followed except for the  
131 final step; RNase from Promega (Charbonnières-les-Bains, France) was added after the lysis step  
132 at 56 °C. As advised by a previous study,<sup>30</sup> the final elution with AE buffer was split into two  
133 elutions of 50 µL to increase the DNA concentration of the eluate. Finally, DNA concentration  
134 was measured with a DS-11 Spectrophotometer from Denovix (Wilmington, NC, USA). DNA  
135 purity of the samples was checked regarding the  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  ratio. DNA samples were  
136 conserved at +4 °C for a maximum of one week. On the day of analysis, all sample concentrations  
137 were set to a maximum concentration of 15 µg mL<sup>-1</sup>, as required by Roche Diagnostic (Meylan,  
138 France), the manufacturer of the qPCR master mix, and 10-fold DNA dilutions were achieved on  
139 8-log scale, down to 0.15 pg mL<sup>-1</sup>.

#### 140 **Design of degenerate oligonucleotides**

141 Design of degenerate oligonucleotides involved six steps (Figure S1). First, data concerning  
142 coding sequences of the *torA* gene from the genera *Shewanella*, *Photobacterium* and *Vibrio* were  
143 retrieved from GenBank and EMBL using SeqinR package with R 3.0.0 (Algorithm S1).<sup>31</sup>  
144 Redundancy in the recovered sequences was checked using the non-redundant databases (NRDB)  
145 program<sup>32</sup> to ensure that there was no duplicate sequence in the dataset. This first step lead to the  
146 selection of 58 *torA* coding sequences comprising 9 sequences of *Shewanella*, 9 of  
147 *Photobacterium* and 40 of *Vibrio* (Table S1). Alignment of *torA* sequences shown heterogeneity

148 among species. This fact and the willingness of developing a qPCR able to amplify the maximum  
149 number of *torA* sequences from different species lead to decide to design degenerate primers and  
150 use them in SYBR green qPCR, although it offers potentially less specificity than the use of  
151 probes. Two sets of suggested degenerate primers, forward and reverse, were obtained for each  
152 bacterial genus thanks to software developed by ProBioGEM laboratory (Lille, France). Briefly, as  
153 described in a previous publication,<sup>33</sup> after alignment, this software calculates the hybridisation  
154 coefficient (HyC). HyC value depends directly from the number of degenerate nucleotides and the  
155 number of possible combinations. It is reflecting the hybridisation ability of the primer: the lower  
156 the value, the greater the probability of specific annealing will be. For this purpose, primers size  
157 and the lower and upper limits of the Hyc were set as follows: 20 bp primers with HyC between 1  
158 and 32 to avoid non-specific hybridisations. The sets of primers were then screened with an  
159 algorithm developed specifically (Algorithm S2) in R 3.0.0 following the usual prerequisites for  
160 the design of oligonucleotides.<sup>34</sup> Retained primers were then tested against initial sequences with  
161 AmplifX v.1.6.2<sup>35</sup> and results were collected in an *ad hoc* database allowing an improved and  
162 reliable dynamic comparison of primer pairs and theoretical PCR products. The first letter of  
163 primer identifiers corresponds to the first letter of genus, e.g. S for *Shewanella*, and the second  
164 letter, F or R, corresponds to the type of primer: forward or reverse. Primers selected after  
165 consulting the database were then analysed using the Oligoanalyzer 3.1 program<sup>36</sup> to check their  
166 propensity to form secondary structures. The limit of primer dimer tolerance concerning binding  
167 energy was fixed as follows: down to -3 kcal/mol for hairpin, -5 kcal/mol in the 3' region, and -6  
168 kcal/mol in the internal region of the primer for self and cross-dimer. In regard to the risk of cross-  
169 dimer formation, two approaches were used regarding both intra-species cross-dimer (e.g. SF  
170 against SR) and inter-species cross-dimer (e.g. SF with PF, PR, VF and VR). Finally, primers were  
171 compared with *M. merlangus* (taxid: 8058) sequences in BLAST. Once design and screening were  
172 completed, desalted primers were synthesised by Sigma-Aldrich (Saint-Quentin Fallavier, France).

### 173 **Amplification conditions**



174 Amplifications were performed in a final volume of 20  $\mu\text{L}$  using a LightCycler 480 system II from  
175 Roche Diagnostics (Meylan, France). 5  $\mu\text{L}$  of template DNA was amplified with pairs of primers.  
176 The LightCycler® 480 SYBR Green I Master kit from Roche Diagnostics (Meylan, France) was  
177 used with final concentration of primers of 0.5  $\mu\text{M}$  and 1  $\mu\text{M}$ . DNA samples, primers and master  
178 mix were dispensed into 96-well white microplates from Roche Diagnostics (Meylan, France).  
179 The initial denaturing step was carried out at 95 °C for 10 min. The amplification cycle was 95 °C  
180 for 30 s, annealing for 30 s and elongation at 72 °C for 30 s. The cycle was repeated 45 times.  
181 Annealing temperatures of 62 °C and 64 °C were tested to avoid non-specific hybridisation, which  
182 can easily occur with degenerate primers. Finally, a melting curve was plotted from 60 °C to 95 °C  
183 with a slope of 0.11 °C.s<sup>-1</sup>.

#### 184 **Post qPCR controls**

185 At the end of qPCR, each PCR product, mixed with the appropriate volume of 6X gel loading dye  
186 from New England Biolabs (Evry, France), were ran in a 1.2% agarose - Tris EDTA Buffer  
187 provided by Eurobio (Les Ulis, France), together with a New England Biolabs 100 bp DNA  
188 ladder. Forward and reverse sequencing were performed by Genoscreen (Lille, France) either  
189 directly on the PCR product or on band extracts from the gel by Wizard SV Gel and PCR Clean-  
190 Up System from Promega (Charbonnières-les-Bains, France), depending on the quality of  
191 electrophoresis pattern. Consensus of forward and reverse sequences were obtained using the  
192 CAP3 algorithm.<sup>37,38</sup>

#### 193 **Preparation of modified atmosphere packed fillets**

194 Twenty-one whole gutted whiting were bought at a local fish wholesaler in Boulogne-sur-Mer  
195 (France), acquired from the morning auction to guarantee a better freshness grade. Initial good  
196 qualities of fish were estimated by looking at various parameters such as skin, eyes and gills. Fish  
197 were caught during spring, in the north-east Atlantic and packed with ice into insulated  
198 polystyrene boxes; unfortunately no precise date of catch was given by the retailer. Fish were  
199 processed at the *PFI-Nouvelles Vagues* innovation platform (Boulogne-sur-Mer, France). The two

200 fillets of each fish were skinned and placed in a unique container equipped with a piece of blotting  
201 paper. The 21 containers were then each placed in a T200 tray-sealer from Multivac (Lagny-sur-  
202 Marne, France) for modified atmosphere packing. The modified atmosphere was achieved by  
203 creating a vacuum down to 10 kPa, and injecting Aligal 15 gas, CO<sub>2</sub> (50%) and N<sub>2</sub> (50%), to a  
204 pressure of 90 kPa. Finally, the containers were thermally sealed with a plastic film. Containers  
205 were then placed in a Binder climatic chamber (Tuttlingen, Germany) at 1 ± 1 °C throughout the  
206 shelf-life study. The day the fillets were prepared was considered as D01 of the study.

### 207 **Fish shelf-life assessment**

208 The qPCR method was applied for the assessment of modified atmosphere packed, whiting fillets  
209 throughout shelf-life. Three containers, each containing the fillets of a single fish, were retrieved  
210 from the climatic chamber on days D01, D03, D06, D08, D10, D13 and D15. qPCR analysis was  
211 performed on each container as described below and, simultaneously, total viable count (TVC),  
212 total volatile basic nitrogen (TVB-N) and trimethylamine (TMA) assays were carried out, serving  
213 as a reference for monitoring the overall microbial load and freshness grade of the fillets. TVC,  
214 TVB-N and TMA measurement methods were performed as previously reported.<sup>27, 39</sup> Briefly,  
215 TVB-N and TMA analysis were performed by acid titration on a deproteinized, steam distilled  
216 extract. TVC was achieved on plate count agar according to the ISO 7218 standard.

### 217 **Fillet preparation for analyses**

218 The process described below was the same for each container. The container was opened under  
219 sterile conditions and the two fillets were cut into approximately 1 cm edge cubes that were mixed  
220 to obtain a representative matrix of the overall state of fish flesh. 25 g of mixed flesh cubes were  
221 reserved for microbiological and molecular analysis and 100 g for TVB-N/TMA assays.  
222 Concerning molecular analysis, a 10-fold diluted sample was used, consisting of 25 g of flesh  
223 cubes stomached in 225 mL of buffered peptone water solution. Two mL of the filtrate were  
224 distributed in cryotubes and conserved in liquid nitrogen until DNA extraction, performed for all  
225 samples five days after the 15<sup>th</sup> day of shelf-life. Before DNA extraction, 1.5 mL of the filtrate was

226 centrifuged at 5000 g for 10 min. The supernatant was then eliminated and extraction was  
227 performed on the pellet as described above. PCR amplification was carried out as previously  
228 described with primer concentrations of 1  $\mu$ M and an annealing temperature of 62 °C.

## 229 RESULTS AND DISCUSSION

### 230 Degenerate primer design and selection *in silico*

231 The first step of primer design, involving the calculation of HyC leads to lists of potential forward  
232 and reverse primers for each bacterial genus. Thus, 1667 forward and reverse primers were  
233 suggested from the alignment of *torA* sequences of *Vibrio*, the majority having HyC values lower  
234 than 16. Concerning *Shewanella*, the software revealed 546 forward and reverse primers with also  
235 a majority of HyC values under 16. Regarding the alignment of *Photobacterium torA* gene  
236 sequences, 395 forward and reverse primers were found for HyC values ranging from 2 to 32. The  
237 subsequent screening using R software, used to ensure that primers met the usual conditions for  
238 primer design, considerably reduced the lists of potential primers, with a percentage of primer  
239 suppression between 88.2% and 95.7%, depending on the lists.

240 Using AmplifX software, more than 13,600 potential amplicons (from 100 to 700 bp) were  
241 suggested for the 58 *torA* gene coding sequences used for primer design, using the reduced list of  
242 primers created with the screening step described above. By setting up a database it was possible  
243 to consign all the potential amplicons and to facilitate the selection process. A first list of primers  
244 suitable for the study of secondary structure formation risk was established (Table 2), based on  
245 their ability to theoretically give PCR products in a majority of the 58 *torA* gene coding  
246 sequences.

247 None of the primers displayed presented a risk of hairpin formation; however six primers showed  
248 a self-dimer formation risk (Table 2). At this stage, the six most promising pairs were preserved  
249 for further investigation (Table 2). None of these primers led to proven homology with *M.*  
250 *merlangus* sequences in BLAST (data not shown). Regarding the risk of formation of intra-species  
251 cross-dimer, only SF34 x SR288 is able to produce a dimer (Table 2). Concerning inter-species

252 cross-dimer, seven primers risk forming secondary structures (Table 2), that is why the multiplex  
253 approach was reconsidered and the following studies were focused on a genus-specific primer pair  
254 selection.

### 255 ***In vitro* characterisation of primer pairs**

256 In a first step, each primer of the six promising pairs was screened for their ability to auto-amplify  
257 under qPCR conditions, using the melting curve as a marker of dimer formation (data not shown).  
258 This step led to the exclusion of PF23xPR167, SF34xSR288 and VF351xVR572, as a clear peak  
259 appeared in the melting curves of PR167, SR288 and VR572 (Table 2). The three last pairs were  
260 used to amplify bacterial DNA, although it was noted that PR168 and SF240 should not be used at  
261 a final concentration above 0.5  $\mu\text{M}$  as secondary structures were observed at 1  $\mu\text{M}$ . Among these  
262 pairs, PF23xPR168 displayed the expected PCR product at 200 bp for *P. phosphoreum* and *P.*  
263 *damselae*; unfortunately, more than ten different and non-specific bands were observed for  
264 *Shewanella* and *Vibrio* species. Similarly, SF140xSR36 gave poor results for each species with  
265 numerous and non-specific bands and absence of PCR product at the expected size of 450 bp.  
266 More interestingly, at 1  $\mu\text{M}$  VF239xVR353 gave a single band at the expected size of 420 bp for  
267 *Vibrio* and *Photobacterium* genera (Table 1), and a single band was also observed for  
268 *S.putrefaciens* but of a smaller size. After this first characterisation, VF239xVR353 was the only  
269 pair selected for optimisation of the conditions for amplification, sensitivity and efficiency testing.

### 270 **Primer pair efficiency, qPCR condition adjustments and sensitivity**

271 The efficiency and the sensitivity of the VF239xVR353 pair was assessed by amplifying 10-fold  
272 dilutions of DNA extracts of *P. phosphoreum*, *P. damsela*, *V. vulnificus* and *V. alginolyticus*.  
273 Results obtained with two primer concentrations and two different annealing temperatures are  
274 given in Table 3. Among all the above-mentioned conditions, the  $R^2$  values were higher than 0.99  
275 from 75 ng to 75 pg of template DNA. Special care was paid to target DNA quality and only fresh  
276 DNA extracts were used for qPCR analysis; indeed after several freezing/thawing cycles of DNA  
277 samples, a Cq drift was observed for the more highly-diluted samples (data not shown). The

278 conditions giving the best amplification efficiency were 62 °C for annealing temperature with  
279 primer concentrations of 1 µM. The mean efficiency values were respectively 92.6%, 91.6%,  
280 92.6% and 93.0% for *P. phosphoreum*, *P. damsela*, *V. vulnificus* and *V. alginolyticus*. Thus,  
281 despite a size longer than the average PCR products described in the literature, the use of this  
282 primer pair allowed to produce an amplicon with acceptable efficiencies with regard to the  
283 different tested species. Finally, the estimated limit of detection of the primer pair for all the  
284 strains is around 75 pg of template DNA, as it was the last quantity of DNA where clear  
285 differences of Cq were observed with the decrease of DNA quantity (Figure S2).

### 286 **Primer pairs selectivity**

287 Results of the selectivity test are shown in Table 1. The selectivity approach confirmed that  
288 VF239xVR353 is able to give a unique *torA* PCR product for a wide range of *Vibrio* species. Only  
289 two species out of the eleven tested gave more than one PCR product but this is not a concern for  
290 this study as they were never described in fishery products. Concerning *Vibrio* species, Cq values  
291 ranging from  $10.98 \pm 0.02$  for *V. mimicus* to  $13.98 \pm 0.01$  for *V. fluvialis* were recorded for 75 ng  
292 template DNA. For the rest of the tested species, recorded Cq values were higher than 30, except  
293 for *E. coli* and *P. agglomerans*. Concerning *S. putrefaciens* and *S. baltica*, no single PCR product  
294 pattern was observed. *S. putrefaciens* showed no band in the neighbourhood of 400 bp, whereas *S.*  
295 *baltica* despite three different patterns among the four strains always displayed a band of  
296 approximately 400 bp identified as part of the *torA* gene. For the remaining strains, only *B.*  
297 *subtilis*, *S. enterica*, *C. divergens* and *C. gilleni* displayed a band in the neighbourhood of 400 bp.  
298 Except for *S. enterica*, these PCR products displayed good identities with *torA* sequences of *V.*  
299 *vulnificus* and *S. baltica*, suggesting that these species might harbour closely-related *torA* genes. *E.*  
300 *coli* and *P. agglomerans* displayed wide bands at sizes different from 400 bp, with respective Cq  
301 values of  $29.68 \pm 0.15$  and  $17.84 \pm 0.22$ . Finally, *S. aureus*, *S. epidermidis* and *C. maltaromaticum*  
302 showed no distinct band on the gel electrophoresis.

### 303 **Application to fish shelf-life assessment**

304 After optimisation steps with pure DNA samples, qPCR method was applied, with other freshness  
305 estimation methods, in the course of a shelf-life study on MAP chilled whiting fillets. Throughout  
306 the study, TMA and TVB-N measurements constantly increased with a slow phase from D01 to  
307 D06 and a more consequent increase from D06 to D15 (Figure 1). In addition, it was noted that  
308 these variables are highly correlated ( $r=0.99$ ;  $p$ -value  $< 0.001$ ). Regarding TVB-N analysis, the  
309 first fillet unsuitable for human consumption was analysed at D10. In the following days the  
310 number of fish judged unfit for consumption, increased with two out of three on D13 and all the  
311 fillets on D15. These results suggest that the limit of consumption of MAP chilled whiting fillets  
312 should be in the neighbourhood of D10. TVB-N showed a slight increase throughout shelf-life  
313 compared to fish conserved on melting ice. Conflicting opinions can be found on the use of TVB-  
314 N assay for shelf-life monitoring of MAP fish. Some authors indicated that MAP had no effect on  
315 TVB-N and TMA values, as for whiting and salmon at diverse MAP conditions including one  
316 close to those studied in this work (60% N<sub>2</sub>/ 40% CO<sub>2</sub>).<sup>40</sup> For others, MAP do have effect,  
317 lowering the TVB-N and TMA values, but it was described on a different species of fat fish.<sup>41</sup>  
318 Currently, no QIM scheme has been developed for the evaluation of the studied matrix, so TVB-N  
319 and TMA were, for this study, the most suitable methods for recording the deterioration of fillet  
320 freshness. Nonetheless, sensory analyses will need to be developed for future studies, to better  
321 estimate the limit of consumption. The TVC study led to inconclusive results (Table 4). Indeed,  
322 the average evolution tended to increase between D01 and D06, when the maximum counts were  
323 recorded, and decreased until D15, when the lowest counts of the shelf-life study were obtained.  
324 The above-mentioned results are markedly different from those described in a previous work on  
325 whiting conserved on melting ice.<sup>27</sup> The differences observed could be explained by the  
326 development of non-cultivable bacteria with the ISO 7218 standard growth conditions, in MAP  
327 chilled whiting. As a consequence, the ISO 7218 standard appears not to be suitable for the  
328 quantification of microorganisms in MAP chilled whiting.

329 Regarding the qPCR approach, application of the VF239xVR353 pair to fish samples gave  
330 encouraging results (Figure 2) with a clear decrease of C<sub>q</sub> values from 31.2 at D01 to 20.2 at D10.

331 For D13 and D15 some lower Cq values were recorded, but these showed heterogeneity among  
332 the three fish fillets analysed. Additionally, an abnormally low value was recorded at D06 that  
333 remains unexplained as no atypical value was recorded in the TVB-N / TMA assays; this data was  
334 discarded for the following results. Based on average values, a good linearity of the signal was  
335 observed from D01 to D08 ( $R^2=0.99$ ). Moreover, an encouraging correlation was calculated ( $r = -$   
336  $0.86$ ;  $p$ -value  $< 0.001$ ) from D01 to D13 between the freshness grade indicator (TVB-N) and Cq  
337 values. This correlation even improved in the early phase of storage between D01 and D06 ( $r = -$   
338  $0.96$ ;  $p$ -value  $< 0.001$ ). Concerning TMA concentrations, coming from the metabolism of the *torA*  
339 gene-harbouring microorganisms, similar observations were made with respective correlation  
340 coefficients of  $-0.81$  ( $p$ -value  $< 0.001$ ) and  $-0.92$  ( $p$ -value  $< 0.001$ ). The heterogeneity of Cq  
341 values observed each day was not clearly explained in this experiment, although heterogeneous  
342 initial quality of the whiting could be hypothesised, as this study was carried out on wild fish. A  
343 tendency for signal stabilisation was observed from D10 which could mark the stationary phase of  
344 spoilage flora growth, explaining the increase of TMA while no significant evolution of Cq values  
345 was observed. The marked decrease of Cq value is consistent with the previous work; <sup>27</sup> it could  
346 also be used as an indirect indicator of freshness, although the type of conservation and the  
347 targeted flora were different. Application of this qPCR technique needs to be tested on larger  
348 sampling size to strengthen conclusions from this study and better characterize the variability  
349 among fish. In the meantime, other storage conditions and other fish species should be analysed to  
350 assess the portability of the method.

351 PCR products were inspected on gel electrophoresis (Figure 3), which highlighted a band of  
352 strong intensity at the expected size, located in the neighbourhood of 400 bp for all days of shelf-  
353 life assessment. It should be mentioned that at D01, an additional slight band was observed for  
354 fish 1.

355 In some cases, sequencing gave double sequences with a clear principal sequence signal and  
356 another low secondary signal. Systematic visual inspection of the chromatograms was performed

357 to avoid secondary signal problems. From D01 to D03, sequencing led to only 76% to 89%  
358 similarity with the *P. phosphoreum* DSM 2167 *torA* gene partial sequence. Sequencing results  
359 showed that PCR products obtained from spoiling whiting from D06 to D15 shared between 89%  
360 and 91% identity with the *P. phosphoreum* DSM 2167 *torA* gene partial sequence amplified by  
361 VF239xVR353. All the PCR products displayed nucleotide sequences very close to each other,  
362 with identity percentage ranging from 97% to 100%. The above-mentioned results led to the  
363 hypothesis that a unique *P. phosphoreum* population outgrew the microbial flora and led to an  
364 increase in quantities of *torA* gene between D06 and D15, confirming previous observations on  
365 the specific spoilage organisms in MAP fish.<sup>21,42</sup> Thus, double sequences recorded mostly in the  
366 early phase of shelf-life could be explained by more than a single microbial species harbouring the  
367 *torA* gene. Another work quantified *P. phosphoreum*, thanks to TaqMan qPCR, during the storage  
368 of farmed turbot (*Scophthalmus maximus*).<sup>43</sup> Contrary to the results presented in our work, *P.*  
369 *phosphoreum* was not detected in turbot. However a lot of parameters could explain this fact, as  
370 Roth *et al.* studied the fish skin, with fish of a different species, which dwell in different habitats  
371 and were stored in different conditions. Additionally, contrary to what is commonly admitted in  
372 qPCR spoilage studies,<sup>44</sup> *luxA*, unlike *torA*, is not a gene directly “related to the spoilage effect”,  
373 as it encodes a luciferase.

374 The qPCR method developed is easily applicable in routine food control, allowing confident  
375 analysis of almost 30 samples within a working day, confirmation on gel not included. That time  
376 can be decreased with the use of automation devices for DNA quantification or automated PCR  
377 preparation. This technique offers an objective analysis tool to assess freshness which is an  
378 advantage over sensory analysis. The qPCR approach allows to potentially analysing a higher  
379 number of samples than with reference chemical analyses. Finally, compared with usual microbial  
380 techniques used in food control, the results are obtained more rapidly.

381 Nonetheless, further developments will be needed to improve the level of reliability of the  
382 observed results. Concerning qPCR new chemistry as SYTO dyes or EvaGreen, better than



383 SYBR, <sup>45, 46</sup> could be tested in a way to assess their impact on efficiencies and reproducibility.  
384 New amplification protocols should be tested using, for example, touchdown qPCR which has  
385 previously been shown to improve specificity. <sup>47</sup> Moreover, as there is very little degeneration in  
386 the primers, substitution by regular primers should be tested to assess the impact of degenerate  
387 primers on specificity. In addition, a new primer pair should be designed for targeting *torA* gene of  
388 *Shewanella* species in order to develop a multiplex approach quantifying all types of fish SSOs.  
389 The results presented in Figure 2 are absolute C<sub>q</sub> values and a way to normalise data should be  
390 developed for future monitoring studies, so as to reduce variability generated by all manipulation  
391 steps. Special care should also be taken in the evaluation of any matrix effect by confirming good  
392 efficiencies and quantitative aspect of the DNA isolation procedure by testing on spiked pauci-  
393 microbial fillets. Any matrix effect could also be evaluated by implementation of controls, such as  
394 samples process control or negative sample process control to improve the reliability analyses of  
395 results. <sup>48</sup> In light of the above-mentioned C<sub>q</sub> values, the number of amplification cycles could  
396 confidently be reduced to 30, thereby reducing the analysis time. As a consequence, the use of an  
397 internal amplification control (IAC) and its integration into the qPCR mix should be considered.  
398 Indeed, IAC has proven to be a good tool to go further in the interpretation of negative results and  
399 for distinguishing true negative results from false negative results caused by PCR inhibition. <sup>49</sup>  
400 Finally, it would be interesting to confirm the results with addition of ethidium monoazide (EMA)  
401 or propidium monoazide (PMA) in a way to solely detect live bacteria.

## 402 CONCLUSIONS

403 Care taken during the primer elaboration step and *in silico* control, together with the use of various  
404 software application, ensured that the design process was reliable. A single pair of primers with a  
405 low number of degenerate nucleotides was selected. This pair was used to amplify a 420 bp region  
406 of the *torA* gene for both *Vibrio* and *Photobacterium*. Applied here for monitoring the shelf-life of  
407 fish, the results suggest that this method would be a great benefit in the field of food control and  
408 fish freshness assessment. This work is the second using the qPCR approach targeting *torA* gene

409 to assess fish freshness, and conclusions are consistent. Concerning Cq values, a decrease was  
410 recorded together with the increase in shelf-life and is correlated with both TVB-N and TMA  
411 evolution. A highly probable outgrowth of *P. phosphoreum*, a specific spoilage organism was  
412 observed from D06. New controls will be necessary to improve the reliability of the results  
413 obtained, before being routinely applied for food control.

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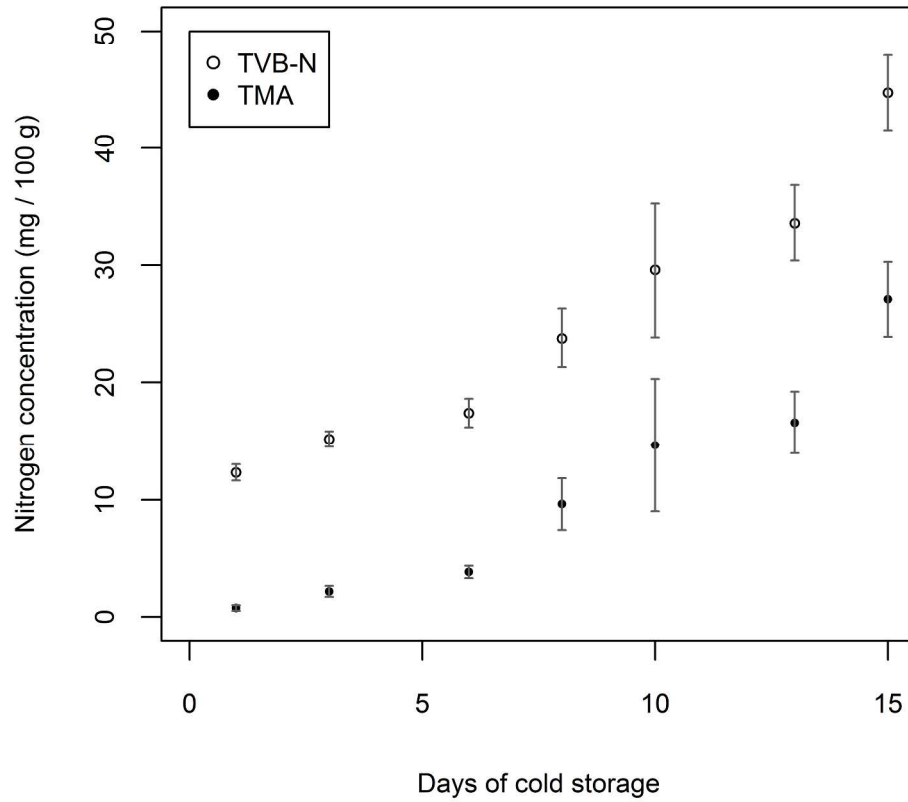


Figure 1: Evolution of TVB-N and TMA concentrations, expressed in nitrogen g per kg of flesh, throughout the shelf-life of fish. Values represent the mean of duplicate measurements assessed on three fish samples and error bars illustrating 95% confidence intervals (2 standard errors).  
127x127mm (600 x 600 DPI)

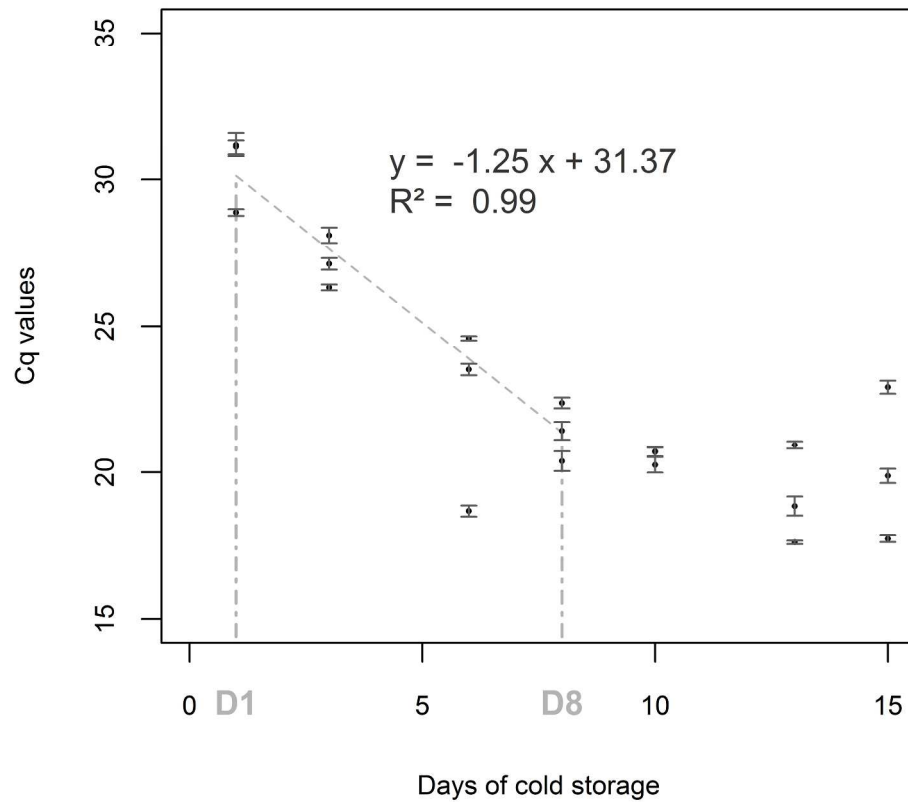


Figure 2: Evolution of Cq values obtained after qPCR amplification of fish fillet DNA extracts, with VR239 x VR353 primer pairs throughout the shelf-life study. Values represent the mean of quadruplicate measurements assessed for a DNA extract from each fillet, and error bars illustrating 95% confidence intervals (2 standard errors). The grey dashed line corresponds to the regression line obtained with the average values from D01 to D08, the atypical value at D06 being discarded.  
127x127mm (600 x 600 DPI)

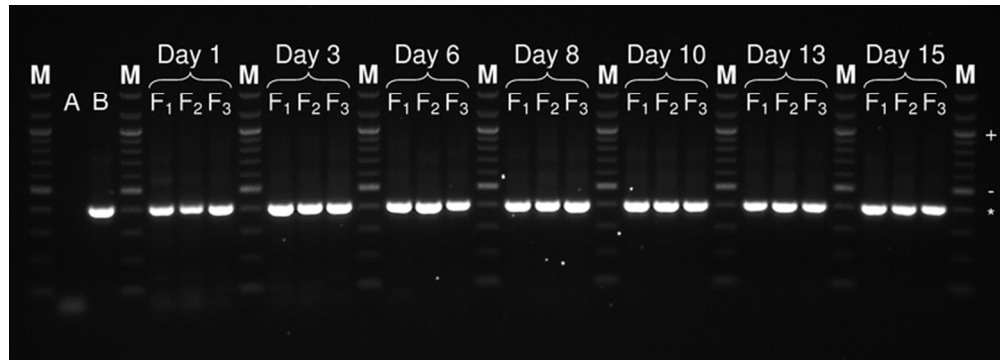


Figure 3: Gel electrophoresis performed with PCR products amplified from DNA extracts from fillets. M tagged tracks correspond to molecular size markers. A and B lanes respectively correspond to the non-target control and a positive control consisting of *P. phosphoreum* DNA extract. The daily PCR products are shown for the three fish fillets (F1, F2 and F3). The symbols "+", "-" and "\*" respectively correspond to band sizes of 1000 bp, 500 bp and 400 bp.

69x24mm (300 x 300 DPI)



**Table 1: Bacterial strains used for this study. The use and the result of specificity test by qPCR with VF239 x VR 353 are given for all the strains (Cq, gel electrophoresis patterns and sequencing).**

Strains	Sources of isolation	Supplier <sup>a</sup>	Use <sup>b</sup>	Mean Cq <sup>c</sup>	Band at the expected size <sup>d</sup>	Aspecific bands	Sequencing results <sup>e</sup>
<i>V. cholerae</i> CIP 106974	Human	CRBIP	S	10.98 ± 0.02	Yes	No	100 % identity with <i>V. cholerae</i> IEC224 <i>torA</i> gene
<i>V. parahaemolyticus</i> CNRVC 010089	Fishery products	CNRVC	S	11.77 ± 0.03	Yes	No	99% identity with <i>V. parahaemolyticus</i> VP2007-007 <i>torA</i> gene
<i>V. alginolyticus</i> CIP 109819	Turbot ( <i>Scophthalmus maximus</i> )	CRBIP	O, S	11.11 ± 0.00	Yes	No	99% identity with <i>V. alginolyticus</i> 40B <i>torA</i> gene
<i>V. vulnificus</i> CIP 75.4	Blood sample	CRBIP	O, S	11.26 ± 0.12	Yes	No	97% identity with <i>V. vulnificus</i> CMCP6 <i>torA</i> gene
<i>V. mimicus</i> CIP 101888	Human ear	CRBIP	S	10.93 ± 0.01	Yes	No	99% identity with <i>V. mimicus</i> VM573 <i>torA</i> gene
<i>V. fluvialis</i> CIP 103355	Human feces	CRBIP	S	13.98 ± 0.01	Yes	No	96 % identity with <i>V. furnissii</i> NCTC 11218 <i>torA</i> gene
<i>V. metschnikovii</i> CIP 69.14	Diseased fowl	CRBIP	S	34.22 ± 0.05	Yes	Yes	No sequencing
<i>V. harveyi</i> CIP 103192	Dead luminescing amphipod	CRBIP	S	11.94 ± 0.01	Yes	No	99% identity with <i>V. harveyi</i> ATCC BAA-1116 <i>torA</i> gene
<i>V. hollisae</i> CIP 104354	Human feces	CRBIP	S	29.55 ± 0.06	Yes	Yes	No sequencing
<i>V. furnissii</i> CIP 102972	Human feces	CRBIP	S	12.92 ± 0.01	Yes	No	98 % identity with <i>V. furnissii</i> NCTC 11218 <i>torA</i> gene
<i>V. nigrapulchritido</i> CIP 103195	Seawater enriched with chitin	CRBIP	S	11.44 ± 0.01	Yes	No	88 % identity with <i>V. cholerae</i> IEC224 <i>torA</i> gene
<i>P. damsela</i> CIP 100540	Turbot ( <i>Scophthalmus maximus</i> )	CRBIP	O, S	13.26 ± 0.11	Yes	No	98 % identity with <i>P. damsela</i> CIP 102761 <i>torA</i> gene
<i>P. phosphoreum</i> DSM 2167	Hake ( <i>Merluccius capensis</i> )	DSMZ	O, S	12.20 ± 0.07	Yes	No	99 % identity with <i>P. phosphoreum</i> ANT 220 <i>torA</i> gene
<i>S. putrefaciens</i> DSM 6067	Tainted butter	DSMZ	O, S	36.52 ± 0.11	No	Yes	No sequencing
<i>S. baltica</i> 14LSABSM1SHW	Whiting ( <i>Merlangius merlangus</i> )	Anses	S	30.89 ± 0.01	Yes	Yes	90 % identity with <i>S. baltica</i> BA 175 <i>torA</i> gene
<i>S. baltica</i> 14LSABSM2SHW	Whiting ( <i>Merlangius merlangus</i> )	Anses	S	33.50 ± 0.24	Yes*	Yes	80 % identity with <i>S. baltica</i> OS 185 <i>torA</i> gene
<i>S. baltica</i> 14LSABSM3SHW	Whiting ( <i>Merlangius merlangus</i> )	Anses	S	32.15 ± 0.02	Yes*	Yes	No identification
<i>S. baltica</i> 14LSABSM4SHW	Whiting ( <i>Merlangius merlangus</i> )	Anses	S	32.07 ± 0.21	Yes*	Yes	73% identity with <i>V. vulnificus</i> MO6-24 <i>torA</i> gene
<i>B. subtilis</i> ATCC 6633	Not communicated	ATCC	S	34.18 ± 0.53	Yes	No	75% identity with <i>V. vulnificus</i> CMCP6 <i>torA</i> gene
<i>S. aureus</i> ATCC 25923	Clinical isolate	ATCC	S	37.54 ± 0.73	No	No	No sequencing
<i>S. epidermidis</i> ATCC 12228	Not communicated	ATCC	S	36.65 ± 0.93	No	No	No sequencing
<i>E. coli</i> ATCC 25922	Clinical isolate	ATCC	S	29.68 ± 0.15	No	Yes	No sequencing
<i>S. enterica</i> ATCC 13076	Not communicated	ATCC	S	34.91 ± 1.00	Yes	Yes	98 % identity with <i>S. enterica</i> CT18 hypothetical protein
<i>C. maltaromaticum</i>	Fish industry environments	Anses	S	37.83 ± 0.36	No	No	No sequencing
<i>C. divergens</i>	Fish industry environments	Anses	S	34.92 ± 0.25	Yes	No	96 % identity with <i>V. vulnificus</i> CMCP6 <i>torA</i> gene
<i>C. gilleni</i>	Fish industry environments	Anses	S	31.16 ± 0.20	Yes	Yes	93 % identity with <i>S. baltica</i> BA175 <i>torA</i> gene
<i>S. proteamaculans</i>	Fish industry environments	Anses	S	32.82 ± 0.01	No	Yes	No sequencing
<i>P. agglomerans</i>	Fish industry environments	Anses	S	17.84 ± 0.22	No	Yes	No sequencing
<i>P. fluorescens</i>	Fish industry environments	Anses	S	34.01 ± 0.09	No	Yes	No sequencing

<sup>a</sup> Suppliers : CRBIP, Centre de Ressources Biologiques de l'Institut Pasteur (Paris, France); CNRVC, French National Reference Centre for Vibrios and Cholera (Paris, France), DSMZ German Collection of Microorganisms and Cell cultures (Leibniz, Germany); Anses, Laboratory's own collection; ATCC, American Type Culture Collection (Manassas, VA, United States). <sup>b</sup> Use of the strain: S for specificity study and O for PCR optimisation. <sup>c</sup> Mean of Cq values recorded for duplicate measures, for 75 ng of template DNA. <sup>d</sup> Existence of a band close by the expected size of 420 bp, values tagged with an asterisk highlight a weak signal of the band. <sup>e</sup> Percentage of identification achieved thanks to BLAST; "no sequencing" is either marked for strains displaying no band at the expected size, or for band not sequenced because of the presence of another band close by; "no identification" refers to a highly weak band that did not allow to recover enough material after extraction.

Table 2: Nucleotide sequences of primers obtained from the first steps of design and sizes of the theoretical PCR products for each pair. Reasons and steps of rejection are communicated when appropriate for each pair.

ID <sup>a</sup>	Forward primers Sequences (5'-3') <sup>b,d,e</sup>	HyC <sup>c</sup>	ID	Reverse primers Sequences (5'-3') <sup>b,d,e</sup>	HyC	PCR products sizes	Rejection step	Reason
PF23	<b>HCGTAA YGTNGATGAAGGTC</b> *	24	PR167	<b>TCTTGR TGGTGR TGCCAWGG</b> *	8	201 bp	<i>In vitro</i>	<i>In silico</i> self-dimer risk confirmed
			PR168	<b>ATCTTGR TGGTGR TGCCAWG</b>	8	202 bp	<i>In vitro</i>	Aspecific PCR products
SF140	<b>ARGGYAARATCCGCGTGATC</b> *	8	SR36	<b>KGAGTAGTGGTGACCATAGC</b>	2	449 bp	<i>In vitro</i>	Aspecific PCR products
			SR37	TKGAGTAGTGGTGACCATAG	2	450 bp	<i>In silico</i>	Self-dimer risk for the forward primer
SF90	YGACTACTCMACMGGTGCGAG	8	SR156	CCACAGGRTCKATRCTGATC	8	251 bp	<i>In silico</i>	Self-dimer risk for the forward primer
SF224	YGGYATYAGCTATGGTCACC	8	SR154	ACAGGRTCKATRCTGATCAC	8	419 bp	<i>In silico</i>	Self-dimer risk for the forward primer
SF34	<b>TYAGCTATGGTCACCACTAC</b> *	2	SR288	<b>YGCCRTASACGTCGATATCG</b>	8	414 bp	<i>In silico</i>	Self-dimer risk for the reverse primer
			VR353	<b>CTCTTGACGYTGGATACACC</b> *	2	249 bp	<i>In vitro</i>	Cross dimer risk, self-dimer risk confirmed
VF239	<b>GCAAGTRGGTTGGAAGTGTG</b>	2	VR349	TGACGYTGGATACACCAACC	2	424 bp	-	Single PCR product
			VR350	TTGACGYTGGATACACCAACC	2	420 bp	<i>In silico</i>	Low number of sequences potentially amplified
			VR419	TCTTGATGGTGGTGCCAWGG	2	421 bp	<i>In silico</i>	Low number of sequences potentially amplified
VF351	<b>TTGGTGTATCCARCCTCAAG</b> *	2	VR572	<b>RTCTTGATGGTGGTGCCAWG</b> *	2	369 bp	<i>In silico</i>	Self-dimer risk for the reverse primer
			VR572	<b>RTCTTGATGGTGGTGCCAWG</b> *	4	370 bp	<i>In vitro</i>	Self-dimer risk for the reverse primer
			VR536	GAATMGTRCGGCTGTAAACCG	4	239 bp	<i>In silico</i>	Self-dimer risk for the reverse primer
VF203	CTGTRGGTATGCAYGGTAAC	4	VR240	TCACAGTTCACCCYACTTG	2	202 bp	<i>In silico</i>	Low number of sequences potentially amplified

<sup>a</sup> Primers identifiers. <sup>b</sup> Nucleotides sequences with degenerations indicated according to IUPAC nomenclature. <sup>c</sup> HyC values calculated according to the above mentioned reference (Tapi, *et al.*, 2010). <sup>d</sup> Primers tagged with an asterisk possibly involved in interspecies cross-dimer formation if a multiplex approach have been developed. <sup>e</sup> The six most promising pair are shown in bold.

**Table 3: Data of standard curves obtained from the four *Vibrio* and *Photobacterium* species. Two temperature values and two primer concentration were tested.**

		0,5 $\mu$ M		1 $\mu$ M	
		62°C	64°C	62°C	64°C
<i>P.phosphoreum</i>	Slope <sup>a</sup>	-3.6794	-3.8335	-3.5118	-3.5387
	Intercept <sup>a</sup>	39.1	41.4	37.3	38.4
	R <sup>2</sup> <sup>a</sup>	0.999	0.998	0.998	0.998
	Efficiency <sup>b</sup>	87.0 %	82.3 %	92.6 %	91.7 %
<i>P.damselae</i>	Slope <sup>a</sup>	-3.6152	-3.8125	-3.5422	-3.6314
	Intercept <sup>a</sup>	38.6	40.0	37.8	38.8
	R <sup>2</sup> <sup>a</sup>	0.996	0.996	0.998	0.999
	Efficiency <sup>b</sup>	89.1 %	82.9 %	91.6 %	88.5 %
<i>V.vulnificus</i>	Slope <sup>a</sup>	-3.8015	-4.0299	-3.5123	-3.7739
	Intercept <sup>a</sup>	39.2	41.2	37.1	38.5
	R <sup>2</sup> <sup>a</sup>	0.999	0.994	0.993	0.999
	Efficiency <sup>b</sup>	83.3 %	77.1 %	92.6 %	84.1 %
<i>V.alginolyticus</i>	Slope <sup>a</sup>	-3.7382	-3.9787	-3.5026	-3.8101
	Intercept <sup>a</sup>	38.1	40.1	36.7	38.5
	R <sup>2</sup> <sup>a</sup>	0.999	0.995	0.996	0.999
	Efficiency <sup>b</sup>	85.1 %	78.4 %	93.0 %	83.0 %

<sup>a</sup> Values were obtained from four replicates on 7 log DNA dilutions ranging from 75 ng to 75 fg of template DNA in the tube. <sup>b</sup> Efficiency was calculated with the formula given by European Network of GMO testing (European Network of GMO Laboratories, 2008).

**Table 4: Evolution of total viable count (CFU log / g) of modified-atmosphere packed whiting fillets, throughout the shelf-life on cold storage.**

	Fish 1	Fish 2	Fish 3
Day 1	4.81 ± 0.09	4.87 ± 0.08	5.16 ± 0.07
Day 3	5.08 ± 0.07	5.10 ± 0.08	5.24 ± 0.06
Day 6	5.46 ± 0.05	5.41 ± 0.05	6.13 ± 0.07
Day 8	5.10 ± 0.05	4.81 ± 0.07	5.16 ± 0.04
Day 10	5.10 ± 0.05	5.43 ± 0.03	5.42 ± 0.04
Day 13	4.90 ± 0.06	4.56 ± 0.08	4.98 ± 0.06
Day 15	4.64 ± 0.08	4.56 ± 0.08	4.78 ± 0.07

Values figured above were calculated according to ISO standard 7218:2007 (International Organization for Standardization, 2007). Each mean value is followed by a value illustrating the 95% confidence interval.

For Peer Review

## Monitoring the freshness of fish: development of a qPCR method applied to MAP chilled whiting

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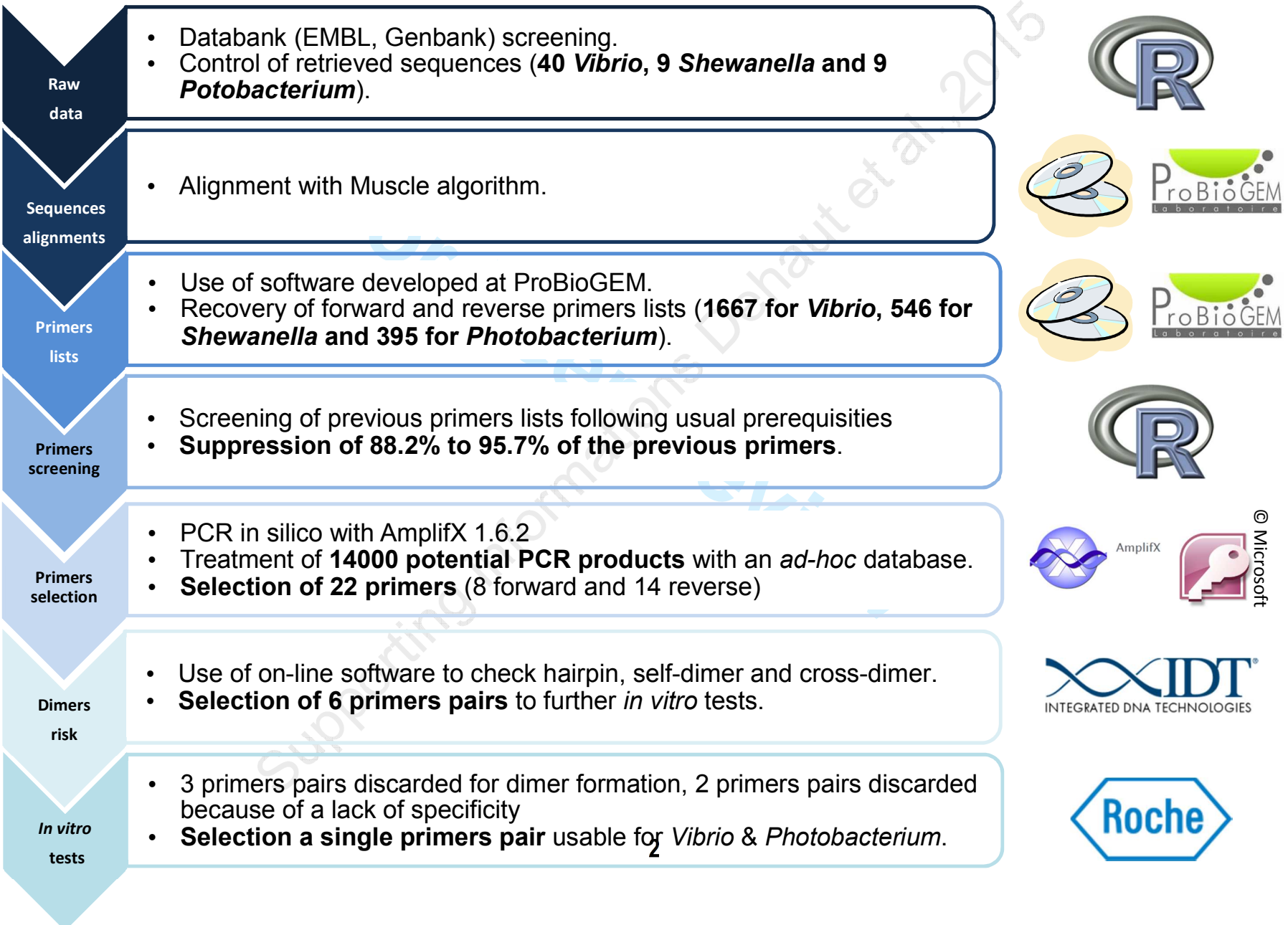
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## Supporting information file

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Figure S1: Workflow of degenerate oligonucleotide design and selection



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## Algorithm S1: Sequence retrieval Algorithm

```

##### A.DEHAUT 2015 #####
#####ENTRIES#####

#Instruction to avoid proxy problems
setInternet2()

#Working directory
setwd('X:/Software/R/Recupseq 2.0')

#Definitions of research parameters
gene<-c("torA")
genus<-c("vibrio","photobacterium","shewanella")
bank<-c("genbank","embl","emblwgs")
type_sequence<-c("cds")
EC_number<-c("1.6.6.9")
#####
#Working packages loading#
library(seqinr)

#Vector length calculation for loops
s_bank<-length(bank)
s_gene<-length(gene)
s_genus<-length(genus)
s_type_sequence<-length(type_sequence)

for (compt_bank in 1 : s_bank)
{
bank[compt_bank]

#Data bank assignation
choosebank(bank[compt_bank])

for (compt_genus in 1:s_genus)
{
for (compt_gene in 1:s_gene)
{

#Request writting
kry<-paste("sp=",genus[compt_genus]," AND ","k=",gene[compt_gene]," AND
","t=",type_sequence,sep = "")

#Creation of research tag
output<-paste(bank[compt_bank],'_ ',genus[compt_genus],sep = "")

#Request creation (Research tag + Request)
query<-query(output,kry)

#Accession and DNA sequence recovery
names<-getName(query)
sequences<-getSequence(query)
nb_access<-query$nelem

#Generation of an output file
fileout<-paste(output,"_",gene[compt_gene],".fasta",sep="")

# File writing in function of accession number
for (compt_access in 1:nb_access)
{

#File with the file recovered
write.fasta(sequences[compt_access],names[compt_access],nbchar=80,file.out=fileout,
open = "a")
#File with all files
write.fasta(sequences[compt_access],names[compt_access],nbchar=80,file.out="whole.f
asta",open = "a")
}}}}

```

Table S1: Accessions corresponding to retrieved sequences

Genera	Accessions
<i>Shewanella</i> :	AP011177 (3680724..3683216); AJ006085 (3702..6191); AE014299 (1282266..1284755); CP000851 (4084665..4087157); CP000931 (4159330..4161828); CP000961 (4303077..4305569); CP000821 (4087725..4090217); CP000606 (3312522..3315008); AJ005974;
<i>Photobacterium</i> :	ADBS01000001 (1639115..1641538); CR378667 (269442..271910); AAOJ01000002 (448268..450736); ADBS01000001 (1581195..1583675); AAOU01000006 (68797..71265); DF093596 (708647..711127); CR378670 (285194..287671); ADBS01000001 (1429059..1431590); HQ599848 (8930..11461)
<i>Vibrio</i> :	AAUR01000023 (26120..28582); AAUU01000010 (33373..35835); AAWF01000013 (33795..36257); AAWP01000118 (1588..4050); AAWQ01000016 (1518..3980); ACYU01000186 (29202..31664); ACYV01000012 (23545..26007); ACZB01000065 (18789..21251); ACZC01000014 (137538..140000); AFOQ01000009 (1055834..1058305); AFOR01000015 (232246..234708); AFOT01000135 (13798..16260); AFOU01000213 (5455..7917); AJRP01000052 (82432..84894); AJSQ01000143 (5890..8352); AJSS01000095 (136403..138865); ALDB01000139 (14508..16970); ALEC01000007 (1007755..1010217); ALED01000026 (1010917..1013379); ACFQ01000010 (768360..770822); ACHV01000001 (403446..405908); ACHW01000019 (67138..69600); ACHY01000017 (241868..244330); ACZN01000018 (875029..877491); ACZP01000013 (351551..354013); ACZT01000023 (256441..258903); ACZV01000005 (486522..488984); ADAF01000001 (1109612..1112074); ADAJ01000008 (1073998..1076460); ADAL01000239 (29982..32444); ADB01000013 (185366..187828); AE016795 (2962329..2964791); ACFM01000025 (10359..12821); CP001805 (2456402..2458864); NC_004459 (2962329..2964791); NC_015633 (1964777..1967239); DS179715 (29829..32291); CP000627 (1377839..1380301); CP001233 (1771825..1774287); CP002469 (1975417..1977879)



## Algorithm S2: Primer Screening Algorithm

```

##### A.DEHAUT 2015 #####

#Working directory assignation#
setwd('X:/Software/R/PrimerAnalysis')

#Package loading#
library(seqinr)

# *.fasta files recovery for the batch treatment#
name<-list.files(pattern = "\\*.fasta$")

#Estimation of the number of file to be treated
l_name<-length(name)

#'for'loop to apply the algorithm at each file
for (compteur_name in 1 : l_name)
{
#File name recovery (standardized name)#
fichier<-name[compteur_name]

#Sliced file to isolate nucleotide (nb_pb) and file name (name_file)#
name_split<-unlist(strsplit(fichier,"-"))

#nb_pb & name_file recovery#
name_file<-unlist(strsplit(name_split[2],"\\.")[1])
nb_pb<-as.numeric(name_split[1])

#Species name recovery#
spc_split<-unlist(strsplit(name_file,"_"))
spc<-spc_split[2]

#Forward or reverse?#
sens<-spc_split[3]

#Sequences of the file recovered and expressed as a vector#
sequ<-read.fasta(fichier)

#All sequences of the file recovered and expressed as a character string#
seq_string<-read.fasta(fichier, as.string=TRUE)

#Recovery of the number of sequences
l_sequ<-length(sequ)

#Matrix initialisation#
tag<-matrix(,l_sequ,1)
sequence<-matrix(,l_sequ,1)
place<-matrix(,l_sequ,1)
hyc<-matrix(,l_sequ,1)
gc_content<-matrix(,l_sequ,1)
gc_end<-matrix(,l_sequ,1)
nb_deg<-matrix(,l_sequ,1)
nb_gc<-matrix(,l_sequ,1)
nb_at<-matrix(,l_sequ,1)
head_tail_inter<-matrix(,l_sequ,1)
gc_content_tail<-matrix(,l_sequ,1)
gc_deg_tail<-matrix(,l_sequ,1)
repet_seek<-matrix(,l_sequ,1)

#'For' loop to treat all the primers contained in the file#

```

```

1   for (compteur in 1 : l_sequ)
2   {
3   #Tag inclusion
4   tag[compteur,]<-paste("Primer_", spc, "_", "_", sens, "_", compteur, sep="")
5
6   #[1] Processed sequence recovery as a character string#
7   sequence[compteur,]<-seq_string[[compteur]][1]
8
9   #Processed sequence recovery as a vector#
10  seq<-sequ[[compteur]][1:nb_pb]
11
12  #[2] Recovery of primer position on the alignment#
13  info_place<-attr(sequ[[compteur]], "Annot")
14  place[compteur,]<-unlist(strsplit(info_place, ":"))[2]
15
16  #[3] Hyc number recovery#
17  info_hyc<-attr(sequ[[compteur]], "name")
18  hyc[compteur,]<-unlist(strsplit(unlist(strsplit(info_hyc, "/"))[1], "="))[2]
19
20  #[4] Determination of G/C number (degenerate nucleotides excluded)#
21  GC<-seq[seq=="g"|seq=="c"]
22  gc<-length(GC)
23  nb_gc[compteur,]<-gc
24
25  #[5] Determination of A/T number (degenerate nucleotides excluded)#
26  AT<-seq[seq=="a"|seq=="t"]
27  at<-length(AT)
28  nb_at[compteur,]<-at
29
30  #[6] Determination of the number of degenerate nucleotides#
31  deg<-nb_pb-(at+gc)
32  nb_deg[compteur,]<-deg
33
34  #[7] Determination of G/C% content estimation (degenerate nucleotides
35  excluded)#
36  pourc_gc<-round(100*(gc/(gc+at)),1)
37  gc_content[compteur,]<-pourc_gc
38
39  #[8] 'if' loop to estimate if the primer sequence is ended by a 'G' or a
40  'C'#
41  if (tail(seq, 1)=="g"|tail(seq, 1)=="c")
42  {
43  gc_end[compteur,]<-c("Yes")
44  }
45  else
46  {
47  gc_end[compteur,]<-c("No")
48  }
49
50  #[9] 'if' loop for estimation of the risk of self-dimerization 5'/3' G/C or
51  C/G#
52  if ((tail(seq, 1)=="g"&&head(seq, 1)=="c")|(tail(seq, 1)=="c"&&head(seq,
53  1)=="g"))
54  {
55  head_tail_inter[compteur,]<-c("Yes")
56  }
57  else
58  {
59  head_tail_inter[compteur,]<-c("No")
60  }
61
62  #G/C number recovery among the 5 last nucleotides in 3'#
63  nb_gc_content_tail<-length(tail(seq, 5)[tail(seq, 5)=="g"|tail(seq, 5)=="c"])

```

```

1  # [10] 'if' loop screening if there is no more than 3 G or C among the 5 last
2  nucleotides in 3' #
3  if (nb_gc_content_tail < 4)
4  {
5  gc_content_tail[compteur,] <- c("No")
6  }
7  else
8  {
9  gc_content_tail[compteur,] <- c("Yes")
10 }

11 # Determination of the nucleotide number in the last 5 nucleotides in 3'
12 (A, T, C, G & W excluded) #
13 control_deg_GC <-
14 tail(seq, 5)[tail(seq, 5) != "a" & tail(seq, 5) != "t" & tail(seq, 5) != "c" & tail(seq, 5) !
15 = "g" & tail(seq, 5) != "w"]

16 # [11] 'if' loop determining if there is no "hidden" G or C (degenerate
17 nucleotides) among the 5 last nucleotides in 3' #
18 if (length(control_deg_GC) != 0)
19 {
20 gc_deg_tail[compteur,] <- c("Yes")
21 }
22 else
23 {
24 gc_deg_tail[compteur,] <- c("No")
25 }

26 # Matrix initialisation to check nucleotide repetition
27 mat_repet <- matrix(, 1, nb_pb)
28 mat_repet[, 1] <- 0
29 mat_repet[, nb_pb] <- 0
30

31 # 'for' loop to screen nucleotides repeat (n-1 et n+1 comparison regarding
32 n) #
33 for (i in 2 : (nb_pb-1))
34 {
35
36 # n comparison to n-1 and n+1 #
37 n_moins <- seq[i] == seq[i-1]
38 n_plus <- seq[i] == seq[i+1]

39 # [12] 'if' loop warning the presence of 3 identical nucleotides repetitions
40 materialized with the value '1' #
41 if (n_moins == "TRUE" & n_plus == "TRUE")
42 {
43 mat_repet[, i] <- 1
44 }
45 else
46 {
47 mat_repet[, i] <- 0
48 }
49 }

50 # Repetition index creation #
51 index_repet <- as.numeric(rowSums(mat_repet))
52

53 # 'if' loop allowing the control of repetitions existence into the primer #
54 if (index_repet == 0)
55 {
56 repet_seek[compteur,] <- c("No")
57 }
58 else
59 {
60

```

```

1   repet_seek[compteur,]<-c("Yes")
2   }
3   }
4
5   #creating a matrix containing all the following parameters
6   [1][2][6][3][4][5][7][8][9][10][11][12]#
7   array_tot<-
8   cbind(tag,sequence,place,nb_deg,hyc,nb_gc,nb_at,gc_content,gc_end,head_tail
9   _inter,gc_content_tail,gc_deg_tail,repet_seek)
10
11  #Recovering the number of column of the above matrix#
12  l_array<-ncol(array_tot)
13
14  #Placing restrictions on various parameters [7][8][5][10][11][12]#
15  array_tot<-
16  subset(array_tot,gc_end=='Yes'&head_tail_inter=='No'&gc_content>40&gc_conte
17  nt<60&gc_content_tail=='No'&gc_deg_tail=='No'&repet_seek=='No')
18
19  #Creation of an export matrix for post processing in Amplifx software
20  array_amplifx<-cbind(array_tot[,2],array_tot[,1])
21
22  #Recovery of the row number in the array#
23  n_row<-nrow(array_tot)
24
25  #Percentage of discarded primers#
26  percent<-round((1-(n_row/l_sequ))*100,1)
27
28  #Construction of a concluding sentence concerning the primer file
29  treatment#
30  ecart<-matrix(,1,l_array)
31  ecart[,1]<-paste(percent,"% des primers ont été écartés grâce à
32  l'algorithm")
33  ecart<-rbind(ecart,matrix(,1,l_array))
34
35  #Title creation#
36  head<-c('ID','Sequence','Situation in the alignment','Number of
37  degeneration','Hyc Number','Number of G/C','Number of A/T','Estimated %
38  GC','End by G or C','Head and Tail Annealing Risk ?','No more than 3 GC in
39  the tail?','No hidden GC (degeneration)in the tail?','No more than 2
40  identical nucleotides near by?')
41
42  #Title and matrice regroupment#
43  array_tot<-rbind(ecart,head,array_tot)
44
45  # *.csv file generation with the above selected informations#
46  array_tot<-as.data.frame(array_tot)
47  export<-
48  write.table(array_tot,file=paste(name_file,".csv",sep=""),dec="," , sep=";",r
49  ow.names = FALSE,col.names = FALSE,na = "")
50
51  #File generation for the automatic treatment with amplifx software#
52  export<-
53  write.table(array_amplifx,file=paste(name_file,"_amplifx.txt",sep=""),sep="
54  \t",row.names = FALSE,col.names = FALSE)
55  }
56
57
58
59
60

```

Figure S2: Efficiency curves

Legend: 62 °C – 1 μM 64 °C – 1 μM 62 °C – 0.5 μM 64 °C – 0.5 μM

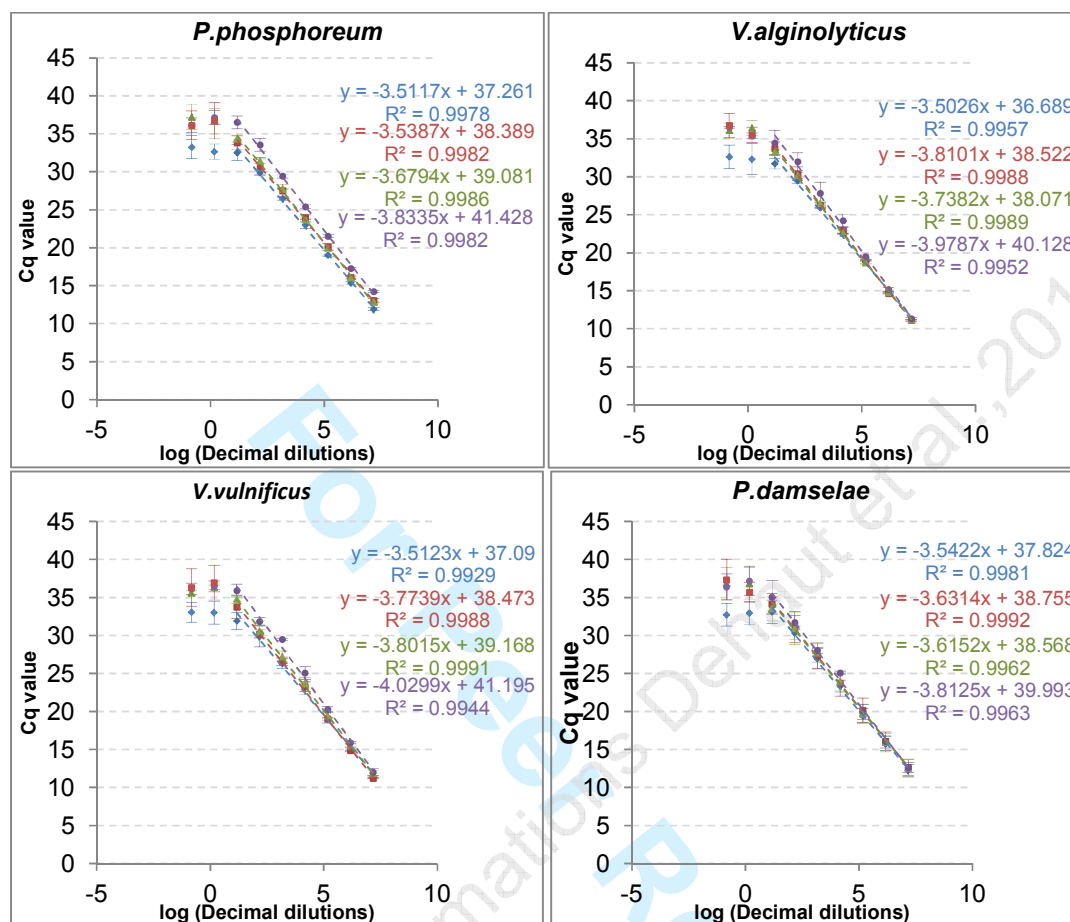
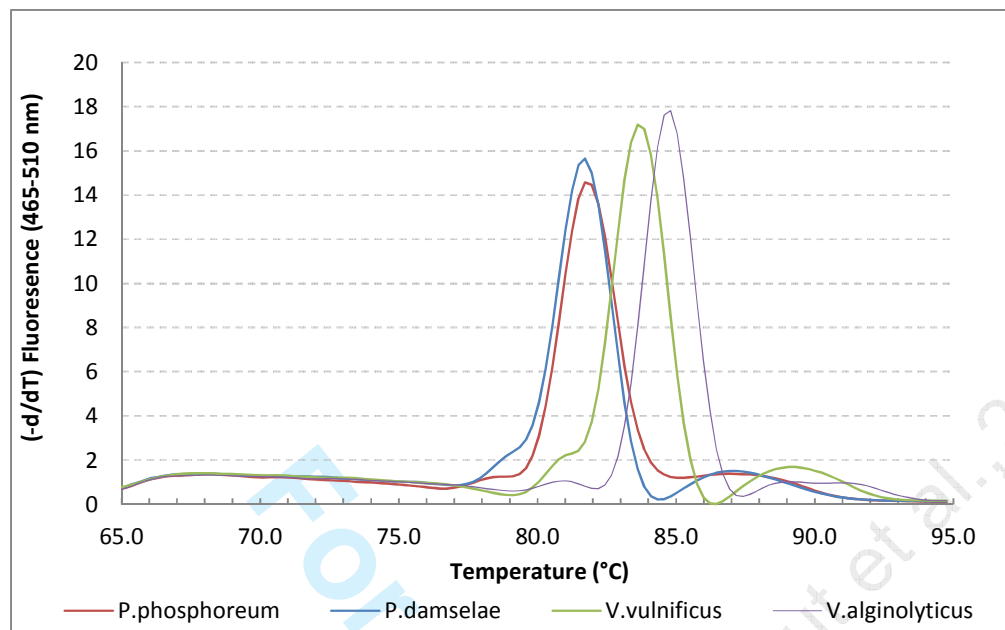


Figure S3: Melting curves



Melting curves obtained for *Photobacterium* and *Vibrio*, with annealing at 62 °C, primers concentration of 1  $\mu$ M and 7.5 ng of DNA.