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Quantification of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* in French Mediterranean coastal lagoons

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Abstract:

Vibrio parahaemolyticus, *Vibrio vulnificus* and *Vibrio cholerae* are human pathogens. Little is known about these *Vibrio* spp. in the coastal lagoons of France. The purpose of this study was to investigate their incidence in water, shellfish and sediment of three French Mediterranean coastal lagoons using the most probable number-polymerase chain reaction (MPN-PCR). In summer, the total number of *V. parahaemolyticus* in water, sediment, mussels and clams collected from the three lagoons varied from 1 to >1.1 × 10^3 MPN/l, 0.09 to 1.1 × 10^3 MPN/ml, 9 to 210 MPN/g and 1.5 to 2.1 MPN/g, respectively. In winter, all samples except mussels contained *V. parahaemolyticus*, but at very low concentrations. Pathogenic (*tdh*- or *trh2*-positive) *V. parahaemolyticus* were present in water, sediment and shellfish samples collected from these lagoons. The number of *V. vulnificus* in water, sediment and shellfish samples ranged from 1 to 1.1 × 10^3 MPN/l, 0.07 to 110 MPN/ml and 0.04 to 15 MPN/g, respectively, during summer. *V. vulnificus* was not detected during winter. *V. cholerae* was rarely detected in water and sediment during summer. In summary, results of this study highlight the finding that the three human pathogenic *Vibrio* spp. are present in the lagoons and constitute a potential public health hazard..

Keywords : *Vibrio* ; Lagoons ; Shellfish ; Water ; Sediment ; Human pathogen

49 **1. Introduction**

50 *Vibrio* spp. are autochthonous to marine and estuarine environments, and are
51 components of those ecosystems (Colwell *et al*., 1977). However, some *Vibrio* species are
52 also human pathogens. *Vibrio parahaemolyticus* is recognized throughout the world as the
53 leading causal agent of human gastroenteritis resulting from consumption of raw seafood.
54 Enteropathogenic strains of *V. parahaemolyticus* generally produce a thermostable direct
55 hemolysin (TDH) and/or a TDH-related hemolysin (TRH). The genes *tdh* and *trh* code for
56 TDH and TRH, respectively (Iida *et al*., 2006). In the United States, *V. vulnificus* is
57 responsible for 95 percent of all seafood-related deaths related to the ingestion of raw or
58 undercooked seafood. Moreover, *V. vulnificus* has often been associated with serious
59 infections caused by exposure of skin wounds to seawater. Different factors have been
60 implicated in virulence of *V. vulnificus* including the *vvhA* gene that encodes hemolytic
61 cytolysin (Oliver, 2006). *Vibrio cholerae*, the causative agent of cholera, has been detected in
62 natural fresh and brackish waters worldwide. This species has also been isolated from areas
63 where no clinical cases of cholera have been reported (Colwell *et al*., 1977). However, most
64 environmental isolates are *V. cholerae* non-O1/non-O139 capable of causing diarrheal
65 outbreaks locally (Rippey, 1994).

66 Vibrios are responsible for many human cases of seafood-borne illness in many Asian
67 countries and the United States (Rippey, 1994; Daniels *et al*., 2000; Su and Liu, 2007). The
68 occurrence of potentially pathogenic *Vibrio* spp. in coastal waters and shellfish of European
69 countries has already been documented, *i.e.*, in Italy, Spain, and France (Barbieri *et al*., 1999;
70 Martinez-Urtaza *et al*., 2008; Hervio-Heath *et al*., 2002). Some non-cholera *Vibrio* outbreaks
71 have also been described in these countries. However, vibrios are rarely responsible for severe
72 outbreaks in Europe but instead for incidence of vibriosis (Geneste *et al.*, 2000). In France,
73 one hundred cases of *V. parahaemolyticus* infection were reported in 2001, all of which
74 involved consumption of mussels imported from Ireland (Hervio-Heath *et al*., 2005). Since
75 then, however, only sporadic cases of *V. parahaemolyticus* infections have been reported
76 (Quilici *et al*., 2005).

77 The coastal lagoons of Southern France (Mediterranean) are ecosystems that receive
78 inputs from watersheds and exchanges with the sea and are thus characterized by significant
79 variation in water temperature and salinity. The coastal area and lagoons, especially Thau, the
80 largest lagoon, are sites of significant shellfish production. *Vibrio parahaemolyticus, V.*
81 *vulnificus,* and *V. cholerae* non-O1/non-O139 were isolated in coastal water and mussel
82 samples collected offshore near the lagoons (Hervio-Heath *et al*, 2002). Two cases of

83 infection involving *Vibrio* spp. have been reported in the south of France. The death in 1994
84 of an immuno-compromised patient was caused by an infection by *V. cholerae* non-O1/non-
85 O139 after exposure of skin wounds to seawater (Aubert *et al*., 2001). In 2008, a fisherman
86 was infected by *V. vulnificus* after a skin injury came into contact with brackish water from
87 the Vic lagoon, in Southern France. This victim, weakened by both kidney and lung failure,
88 died as a result of sepsis (Personal communication).

89 The presence of pathogenic vibrios in these lagoons represents a potential public
90 health threat. To evaluate public health risk, data on the prevalence, distribution, and
91 virulence of these bacteria are needed.

92 In this study, the occurrence and abundance of three human pathogenic *Vibrio* species
93 (*V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*) were investigated in water, shellfish, and
94 sediment samples collected from three coastal Mediterranean lagoons during summer and
95 winter seasons of 2006 and 2007. To our knowledge, this report represents the first detection
96 and quantification of these three *Vibrio* species simultaneously in water, shellfish, and
97 sediment of a lagoon ecosystem.

98 **2. Materials and methods**

99

100 *2.1 Sampling sites*

101

102 Figure 1 shows the location of sampling sites included in this study: Thau, Prévost,
103 and Mauguio, three lagoons on the French Mediterranean coast (Languedoc area). These
104 lagoons were selected on the basis of fishery and recreational activities that take place there.
105 The Thau lagoon is of economical importance due to its large-scale bivalve mollusk farming
106 (approximately 15,000 t of mussels and oysters produced each year), surface area of 75 km^2,
107 and mean depth of 5 m. Small-scale recreational activities (bathing and sailing) also take
108 place in this lagoon. The Prévost lagoon (29 km^2, 0.8 m mean depth) sustains a small shellfish
109 (mussels) production capacity. Unlike the Thau and Prévost lagoons, each of which has
110 salinity similar to seawater, the Mauguio lagoon, with a controlled seawater entry, displays a
111 significantly lower salinity (31.7 km^2, 0.8 m mean depth).

112

113 *2.2. Sample collection and processing*

114

115 Surface water (5 l) and sediment (five 800 cm^3 cores) samples were collected in
116 September, 2006, and January and June, 2007, at one site in each lagoon (Thau: N
117 43°23'35.8'', E 003°37'20.8''; Prévost: N 43°31'16.6'', E 003°54'03.1''; and Mauguio: N
118 43°35'09.5'', E 004°01'15.4'') along with mussels (*Mytilus galloprovincialis,* 20-30 per
119 sample) from the Thau and Prévost lagoons and clams (*Ruditapes decussatus*, 30-40 per
120 sample) from the Thau lagoon. Water temperature and salinity were recorded simultaneously
121 at the time of sampling at each site. Environmental samples were transported in coolers (12-
122 15 °C) to the laboratory and processed within 4 hours of collection.

123

124 *2.3 Quantification of V. parahaemolyticus, V. vulnificus and V. cholerae by MPN-PCR*

125

126 A combined Most Probable Number-Polymerase Chain Reaction (MPN-PCR) method
127 (Luan *et al*., 2008) was applied to detect and enumerate *V. parahaemolyticus*, *V. vulnificus*
128 and *V. cholerae* in the environmental samples. Quantification of the vibrios was achieved by
129 enrichment in alkaline peptone water (APW), following application of the Most Probable
130 Number method. Growth of the *Vibrio* species in APW broth was confirmed by PCR and
131 enteropathogenic *V. parahaemolyticus* (*tdh* positive and *trh2* positive) by real-time PCR.

132 Water samples (1, 10, 100 ml and 1 l) were filtered, in triplicate, through 0.45 µm pore size
133 membranes (nitrocellulose, Whatman, GE healthcare, Versailles, France) and the filters were
134 incubated in APW at 41 °C for 24 h. Superficial sediment samples collected from the first
135 three centimeters of five cores were mixed thoroughly and flesh and intra-valvular liquid of
136 mussels and clams (shellfish tissue) were each homogenized. From the preparations of
137 sediment or shellfish, 10 ml and 1 ml of serial 10-fold dilutions were inoculated in triplicate
138 into APW broth and incubated at 41 °C for 24 h.

139 After enrichment, bacterial DNA was extracted from 1 ml of the APW using the
140 Wizard Genomic DNA Purification kit (Promega, Charbonnières, France) designed for Gram-
141 negative bacteria. Three primer pairs, based on the *toxR* and *vvhA* genes, and a portion of the
142 Intergenic Spacer Region (ISR) 16S-23S rRNA were used to detect *V. parahaemolyticus*, *V.*
143 *vulnificus* and *V. cholerae*, respectively (Table 1). PCR amplification included an initial
144 denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, primer annealing
145 at 57 °C for 30 s, and extension at 72 °C for 30 s, and final extension at 72 °C for 8 min.

146 This protocol was performed in an Eppendorf Mastercycler (Eppendorf, Le Pecq,
147 France) and optimized in a 25 µl reaction containing 5 µl of 5X buffer (Promega,
148 Charbonnières, France), 0.5 µl of dNTPs (200 µM), 0.25 µl of each primer (25 µM)
149 (Invitrogen, Cergy pontoise, France), 13.9 µl of ultrapure water (Millipore SAS, Molsheim,
150 France), 5 µl of target DNA (undiluted, diluted 1/10 and 1/100), 0.1 µl GoTaq DNA
151 polymerase (5 U/µl, Promega, Charbonnières, France), 1 mg/ml of BSA (Sigma-Aldrich
152 Chimie SARL, Saint Quentin Fallavier, France). The PCR-amplified DNA products were
153 separated on a 1.2% agarose gel in Tris-Borate ETDA (TBE) buffer pH 8.3 (Invitrogen,
154 Cergy pontoise, France), at 100 V for 30 min with a 1-Kb Plus DNA Ladder (Invitrogen,
155 Cergy pontoise, France) and revealed with ethidium bromide (0.5 mg/ml).

156 MPN values were calculated from the statistical tables of De Man and expressed as
157 MPN per liter, MPN per milliliter, and MPN per gram, for water, sediment, and shellfish
158 tissue samples, respectively.

159

160 *2.4 Quantification of tdh + and trh2+ V. parahaemolyticus by MPN-real-time PCR*

161

162 *Vibrio parahaemolyticus* (*toxR*) positive enrichment cultures were further
163 characterized by real-time PCR (TAQMAN probe, Eurogentec, Seraing, Belgique) for
164 presence of virulence-associated genes, *tdh* and *tdh*-related hemolysin, *trh2*, found in
165 enteropathogenic *V. parahaemolyticus*. Primers and probes for *tdh* and *trh2* genes selected for

166 real time PCR assay were designed based on the sequences of a 269bp- and 500bp-region of
167 the two genes, respectively, using primers from Bej *et al*. (1999). Sequence data are available
168 on Genbank under accession numbers AF378099 and AY034609 for *tdh* and *trh2*,
169 respectively. The real-time PCR systems developed for these two genes exhibited positive
170 amplification on 8 clinical and 30 environmental *V. parahaemolyticus* strains. TaqMan PCR
171 using *tdh* and *trh2* primers and probes on 50 other bacterial isolates belonging to the *Vibrio*
172 genus (*V. vulnificus*, *V. cholerae*, *V. alginolyticus*, *V. mimicus*) and to other genera
173 (*Aeromonas, Listonella, Citrobacter, Proteus, Klebsiella, Salmonella, Enterobacter,*
174 *Escherichi, Pasteurella* and *Photobacterium*) did not exhibit any amplification, and thus,
175 confirmed the specificity of detection. The sensitivity was tested using real-time PCR on
176 serial-dilutions of genomic DNA purified from *V. parahaemolyticus tdh*+ and *V.*
177 *parahaemolyticus trh2*+ and exhibited amplification of *tdh* and *trh2* genes at the level of 0.33
178 pg and of 0.126 pg, respectively. Alternatively, unenriched 10-fold serial-dilution of pure
179 cultures of *V. parahaemolyticus tdh* and *trh2* exibited a detection level of 1.75 10^2 CFU/ml
180 and of 4 10^2 CFU/ml with the above primers and probes for *tdh* and *trh2*, respectively.
181 Furthermore, the standards used as controls (PCR-positive control) in these assays were
182 plasmids that were cloned with *tdh* and *trh2* amplicons obtained with the real-time systems.
183 The MPN values were calculated and expressed as above.

184 **3. Results**

185

186 *3.1 Vibrio parahaemolyticus*

187

188 *Vibrio parahaemolyticus* was detected in water samples collected from the three
189 lagoons included in this study during the summer months (September 2006, and June 2007)
190 (Fig. 2). Concentrations varied from 1 to 20 MPN/l in the Thau lagoon and 1,100 MPN/l and
191 more in the Mauguio and Prévost lagoons. Water temperatures ranged from 20 °C to 24 °C in
192 the three lagoons and salinity from 36 to 39.6 ‰ in the Thau and Prévost lagoons; Mauguio
193 had lower salinity, 29.6 ‰ in September, 2006, and 20 ‰ in June, 2007. In January, 2007,
194 culturable *V. parahaemolyticus* was detected only in the Prévost lagoon, but at a
195 concentration 1,000 times lower than during the summer months (0.1 to 1 MPN/l). Water
196 temperatures at the time of sampling were 8 °C, 11 °C and 3 °C for the Thau, Prévost and
197 Mauguio lagoons, respectively, and salinity was comparable to summer salinities, *i.e.*, 37 ‰,
198 34 ‰ and 20 ‰, respectively. Except for June, 2007, in Thau, enteropathogenic *trh2+ V.*
199 *parahaemolyticus* was detected in water samples collected from the three lagoons during the
200 summer in numbers from 20 to more than 1,100 MPN/l. Enteropathogenic *tdh+ V.*
201 *parahaemolyticus* was detected only in water samples collected from Thau lagoon (0.4
202 MPN/l) and from Mauguio lagoon (11 MPN/l) in September, 2006. However, no
203 enteropathogenic *V. parahaemolyticus* was detected in water samples collected from any of
204 the lagoons during winter sampling (January 2007).

205 The total number of *V. parahaemolyticus* in all sediment samples collected from the
206 three lagoons varied from 0.04 to 0.4 MPN/ml in winter (January 2007) and during the
207 summer months, varied from 0.09 to 5 MPN/ml, 11 to 110 MPN/ml and 11 to 1,100 MPN/ml
208 in the Thau, Mauguio and Prévost lagoons, respectively. Enteropathogenic *trh2+ V.*
209 *parahaemolyticus* was detected in sediment samples collected from the Mauguio and Prévost
210 lagoons at concentrations of 0.04 to 0.23 MPN/ml in winter and 5 to 210 MPN/ml in summer,
211 but only once in sediment collected from the Thau lagoon (0.9 MPN/ml in September, 2006).
212 Enteropathogenic *tdh+ V. parahaemolyticus* was detected only in September, 2006, in
213 sediment samples collected from the Thau and Mauguio lagoons (0.04 MPN/ml).

214 *V. parahaemolyticus* was consistently detected in shellfish tissue during the warm
215 season (Table 2), with concentrations varying from 9 to 210 MPN/g of mussels and from 1.5
216 to 2.1 MPN/g of clams. While *V. parahaemolyticus* was absent in mussels during the winter,
217 it nevertheless remained detectable in clams (1.5 MPN/g). The concentration of

218 enteropathogenic *trh2+ V. parahaemolyticus* in shellfish tissue was lower than the
219 concentration of total *V. parahaemolyticus*, varying from 0.07 to 9 MPN/g in mussels
220 collected from the Prévost lagoon and detected only once (0.03 MPN/g) in mussels collected
221 from the Thau lagoon (June 2007). Enteropathogenic *trh2+ V. parahaemolyticus* was not
222 detected in clams and was absent from shellfish collected in January, 2007. Enteropathogenic
223 *tdh+ V. parahaemolyticus* was detected in clams sampled during the summer and winter
224 (from 0.07 to 0.4 MPN/g). However, it was detected only once in mussels collected from
225 Thau lagoon in September, 2006 (0.04 MPN/g).
226
227 *3.2 Vibrio vulnificus*
228
229 *Vibrio vulnificus* was detected during the warm season in water samples collected
230 from Mauguio lagoon, varying from 40 to more than 1,100 MPN/l, in water samples collected
231 from Thau lagoon in June, 2007, and from Prévost lagoon in September, 2006 (70 MPN/l and
232 approximately 1 MPN/l, respectively) (Fig. 2).
233 *V. vulnificus* was not detected in sediment samples collected from Prévost lagoon and was
234 detected in Thau lagoon sediment in June, 2007 (0.4 MPN/ml). The concentration of *V.*
235 *vulnificus* ranged from 0.07 to more than 110 MPN/ml during the summer months in Mauguio
236 lagoon sediment samples and was not detected in sediment samples collected from the three
237 lagoons during the winter.
238 *V. vulnificus* was not isolated from mussel samples collected from Prévost lagoon
239 (Table 2), but was detected in clams collected from Thau lagoon during the warm months
240 (between 0.04 to 15 MPN/g), and in mussels from the same lagoon in June, 2007
241 (0.04 MPN/g).
242
243 *3.3 Vibrio cholerae*
244
245 *Vibrio cholerae* was detected in water samples collected from Mauguio lagoon only
246 during the warm season (concentrations ranging from 20 to 40 MPN/l) and from Prévost
247 lagoon in September, 2006 (14 MPN/l) (Fig. 2). It was not detected in water samples
248 collected from Thau lagoon and was detected only in sediment samples from Prévost lagoon
249 in September, 2006 (0.07 MPN/ml). *V. cholerae* was not detected in shellfish collected from
250 the Thau and Prévost lagoons (Table 2). Isolates from *V. cholerae*-positive APW broth
251 streaked onto TCBS agar were confirmed as *V. cholerae* non-O1/non-O139 (data not shown).

252 4. Discussion

253

254 In this study, *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae* were detected and
255 enumerated in environmental samples (water, sediment, mussels, and clams) using the MPN-
256 PCR method. This method was used because it permits enhanced detection of *Vibrio* spp.
257 compared to direct plating using selective media, and notably because large samples can be
258 employed (1-liter water samples inoculated in triplicate and 10 ml in triplicate of sediment or
259 shellfish). Furthermore, the MPN-PCR and MPN-real-time PCR methods were selected
260 because they allowed to provide data comparable to those obtained in studies investigating the
261 presence and ecology of *Vibrio* spp. and pathogenic *Vibrio* species in seafood and coastal
262 environmental samples from many other parts of the world (Wright *et al*., 2007; Luan *et al*.,
263 2008; Blanco-Abad *et al*., 2009; Vezzulli *et al*., 2009).

264 The presence of the three *Vibrio* spp. pathogenic for humans was either not detected in
265 water samples collected from the Thau, Prévost and Mauguio lagoons or detected at very low
266 concentrations during the winter, while higher concentrations were detected during the
267 summer, confirming results of investigators in the United States (Motes *et al*., 1998; Pfeffer *et*
268 *al*., 2003; Parveen *et al*., 2008) and Japan (Fukushima and Seki, 2004). These *Vibrio* spp.
269 have also been detected in European coastal waters, *i.e.* in France (Hervio-Heath *et al*., 2002;
270 Robert-Pillot *et al*., 2004; Deter *et al*., 2010), Spain (Martinez-Urtaza *et al*., 2008), Italy
271 (Barbieri *et al*., 1999), Denmark (Hoi *et al*., 1998), and Norway (Bauer *et al*., 2006).

272 Most of the investigations showed the presence or absence of these bacteria in water
273 samples. However, few studies reported total culturable *V. parahaemolyticus*, *V. vulnificus,*
274 and *V. cholerae*. The counts of culturable *V. vulnificus* ranged from $3x10^4$ bacteria/l to
275 $2x10^5$ bacteria/l in surface waters of Chesapeake Bay (Wright *et al.,* 1996) and from 5 to
276 19 MPN/l in Danish marine waters (Hoi *et al*., 1998). Counts of *V. parahaemolyticus* and *V.*
277 *vulnificus* were $9.3x10^4$ MPN/l in estuarine water samples collected from the Sada River in
278 Japan (Fukushima and Seki, 2004). Concentrations of *V. cholerae* in recreational beach
279 waters of Southern California were < 15 to 60.9 CFU/l, with higher concentrations in
280 tributaries up to $4.25x10^5$ CFU/l (Jiang, 2001). High concentrations of *V. parahaemolyticus*
281 (up to 10^5 CFU/l), *V. vulnificus* (10^4 CFU/l), and *V. cholerae* ($2x10^4$ CFU/l) were reported in
282 estuarine waters of Eastern North Carolina during the warmer season and of the Northern
283 Gulf of Mexico (Pfeffer *et al*., 2003; Zimmerman *et al*., 2007; Blackwell and Oliver, 2008).
284 Depending on the lagoon sampled, the concentrations were a hundred-fold higher than those
285 reported in this study.

286 Temperature has been shown to be the major factor explaining the dynamics of *V.*
287 *parahaemolyticus*, *V. vulnificus,* and *V. cholerae* in coastal marine ecosystems. Many studies
288 have shown, both experimentally and *in situ,* that these bacteria enter a viable but non
289 culturable state when water temperatures average less than 15 °C (Roszak and Colwell, 1987;
290 Colwell and Grimes, 2000). As observed in lagoon water, this phenomenon could explain the
291 absence or presence in very low concentrations of culturable *Vibrio* in marine coastal waters
292 in the winter. Temperatures above 20 °C favor growth of *Vibrio* spp. in seawater (Motes *et*
293 *al*., 1998; DePaola *et al*., 2003; Blackwell and Oliver, 2008). Our results show that
294 temperatures ranging from 20 °C to 24 °C during the summer months in the three
295 Mediterranean lagoons studied were correlated with presence of these bacteria.

296 Salinity is also an important parameter in the dynamics of vibrios in marine systems
297 (Hsieh *et al*., 2008). Many studies have shown a strong correlation between the presence of
298 these three *Vibrio* spp. and temperature and salinity (Colwell *et al*., 1977; Wright *et al*., 1996;
299 Motes *et al*., 1998; Jiang, 2001; DePaola *et al*., 2003; Pfeffer *et al*., 2003; Randa *et al*., 2004;
300 Blackwell and Oliver, 2008). The results indicate that a decrease in salinity favors *Vibrio*
301 growth and proliferation, particularly in brackish waters of estuaries. In this study, the highest
302 concentrations of *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae* occurred in the Prévost
303 and Mauguio lagoons, both of which have lower salinities than the Thau lagoon. A higher
304 abundance of *V. vulnificus* was observed in the Mauguio lagoon, where salinity ranges from
305 20 to 29 ‰, confirming that salinity is a strong determinant of *V. vulnificus* abundance and
306 dynamics, as previously reported by Randa *et al.* (2004).

307 The ecology of *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae* in coastal waters is
308 relatively well documented, but information is scarce for sediments. Vibrios are present in
309 sediment during the summer and are either absent or present in low numbers in the winter
310 (DePaola *et al*., 1994; Pfeffer *et al*., 2003; Fukushima and Seki, 2004). *V. parahaemolyticus*
311 and *V. cholerae* were detected at concentrations up to $3x10^3$ MPN/l and $2x10^2$ MPN/l,
312 respectively, in sediment samples collected from the Spezia Gulf, Italy (Vezzuli *et al*., 2009).
313 The densities of *V. parahaemolyticus* were one hundred times lower than those reported in
314 this study. *V. parahaemolyticus* and *V. vulnificus* counts in estuarine sediment samples
315 collected from the Sada river in Japan displayed values comparable to those observed in
316 sediment samples from the Prévost and Mauguio lagoons (Fukushima and Seki, 2004). *V.*
317 *vulnificus* has also been detected in large numbers in estuarine sediment samples (DePaola *et*
318 *al*., 1994; Wright *et al*., 1996; Hoi *et al*., 1998). Like estuarine sediments, sediment in the
319 lagoons accumulates runoff from the watershed. This watershed discharge supports growth of

320 vibrios. Moreover, *V. parahaemolyticus* and *V. vulnificus* concentrations in the lagoon
321 sediments were, on average, 100 to 1,000 times higher than in the water column. *V. cholerae*
322 was detected less frequently, with equivalent concentrations in sediment and water. Thus, it
323 can be concluded that sediment serves as a reservoir for these *Vibrio* spp. (DePaola *et al.*,
324 1994; Fukushima and Seki, 2004; Randa *et al.*, 2004; Vezzulli *et al.*, 2009). *V.*
325 *parahaemolyticus* is absent from the water column during the winter season, but it is present
326 in sediment, suggesting that sediment allows at least one subpopulation of these bacteria to
327 survive in the culturable state.

328 The number of *Vibrio* spp. in shellfish varies widely and depends on geographical
329 area, environmental conditions, and local parameters. For example, *V. parahaemolyticus* was
330 detected in concentrations ranging from < 10 to 12,000 CFU/g in Alabama oysters (DePaola
331 *et al.*, 2003), < 10 to 600 MPN/g in Chesapeake Bay oysters (Parveen *et al.*, 2008), < 10 to
332 32 MPN/g in mussels collected in Spain (Martinez-Urtaza *et al.*, 2008), < 10 to 10,000 CFU/g
333 in oysters from India (Deepanjali *et al.*, 2005), and < 10 to 1,500 MPN/g in New Zealand
334 oysters (Kirs *et al.*, 2011). In oysters from the lagoons of Mandinga (Veracruz), Mexico, the
335 concentrations of *V. parahaemolyticus* ranged from < 3 to 150 MPN/g (Reyes-Velazquez *et*
336 *al.*, 2010), comparable to the numbers in mussels from the Thau and Prévost lagoons (9 to
337 210 MPN/g).

338 The number of *V. parahaemolyticus* in shellfish is an indication of the potential risk of
339 gastroenteritis following consumption of shellfish. However, quantification of pathogenic
340 (*tdh*- or *trh*-positive) *V. parahaemolyticus* provides perhaps a better estimate of public health
341 risk (Zimmerman *et al.*, 2007). Many studies have detected the two virulence genes (*tdh* or
342 *trh*) in coastal water, oyster, and mussel samples and in environmental isolates of *V.*
343 *parahaemolyticus* (DePaola *et al.*, 2003; Robert-Pillot *et al.*, 2004; Deepanjali *et al.*, 2005;
344 Bauer *et al.*, 2006; Zimmerman *et al.*, 2007; Martinez-Urtaza *et al.*, 2008; Parveen *et al.*,
345 2008; Deter *et al.*, 2010; Kirs *et al.*, 2011). In general, the percentage of samples that were
346 positive for pathogenic *V. parahaemolyticus* varied according to geographic site, ranging
347 from < 20 % to 100 %. However, the percentage of pathogenic *V. parahaemolyticus* strains
348 was < 0.1 % to 15 % of total *V. parahaemolyticus* (Hervio-Heath *et al.*, 2002; DePaola *et al.*,
349 2003; Robert-Pillot *et al.*, 2004; Ottaviani *et al.*, 2010; Deter *et al.*, 2010).

350 Very few data are available on the number of pathogenic *V. parahaemolyticus* in
351 shellfish. The average number of *tdh*+ *V. parahaemolyticus* in oysters collected from two sites
352 in Alabama was 2.7 CFU/g and 1.3 CFU/g, respectively (DePaola *et al.*, 2003). The number
353 of *tdh*+ *V. parahaemolyticus* in oysters in Chesapeake Bay was 10 CFU/g (Parveen *et al.*,

354 2008). In the Northern Gulf of Mexico, the number of *tdh*+ *V. parahaemolyticus* and *trh*+ *V.*
355 *parahaemolyticus* ranged from < 0.01 to 10 MPN/g oyster tissue (Zimmerman *et al*., 2007).
356 The number of pathogenic *V. parahaemolyticus* found in shellfish in this study was slightly
357 lower and reflects the lower concentration of total *V. parahaemolyticus* in shellfish from
358 Mediterranean lagoons.

359 This study is the first to examine simultaneously the concentrations of *V. vulnificus*, *V.*
360 *cholerae* non-O1/non-O139 and both total and pathogenic (*tdh*- or *trh2*+-positive) *V.*
361 *parahaemolyticus* in water, sediment and shellfish in lagoons.

362 The three major pathogenic *Vibrio* spp. for humans were detected in the lagoons and
363 their presence in shellfish mainly eaten raw represents a public health hazard. More
364 information is needed to improve the quantitative risk assessment concerning presence of
365 vibrios in shellfish (WHO, 2011). DePaola *et al.* (2000) requires the densities > 10 of *tdh*-
366 and/or *trh*-positive *V. parahaemolyticus* be considered unusual. It would be important to
367 determine if any physicochemical condition, other than water temperature, favors an increase
368 in *Vibrio* populations. Lagoons with lower salinity or showing a significant decrease in
369 salinity due to heavy rainfall need to be studied to determine the effects of both salinity and
370 temperature, combined, on *Vibrio* population dynamics. Organic matter entering from the
371 watershed to the lagoon during heavy rainfall also may significantly affect the dynamics of
372 these vibrios. In any case, environmental factors certainly play an important role in the
373 dynamics of *Vibrio* spp. and may well provide preventive measures for management of
374 shellfish safety.

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376

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518 **Legends to figures**

519

520 Fig. 1. Location of the Thau, Prévost, and Mauguio lagoons on the French Mediterranean
521 coast (Languedoc area).

522 Fig. 2. Numbers of *Vibrio parahaemolyticus*, *V. parahaemolyticus trh2+*, *V.*
523 *parahaemolyticus tdh+*, *V. vulnificus,* and *V. cholerae* in water and sediment samples
524 collected from the Thau, Prévost and Mauguio lagoons. The units are Log MPN/l for water
525 samples and Log MPN/ml for sediment samples.

526 **Tables**

527 Table 1: Primers used in this study to detect *V. parahaemolyticus*, *V. vulnificus,* and *V.*
528 *cholerae* in enrichment culture.

Vibrio species	Target genes region	Primer sequences[1]	Reference
V. parahaemolyticus	*toxR*	F-toxRvp: 5'-GTCTTCTGACGCAATCGTTG-3' R-toxRvp: 5' -ATACGAGTGGTTGCTGTCATG-3'	Kim et al. (1999)
V. vulnificus	*vvhA*	L-CTH: 5'-TTCCAACTTCAAACCGAACTATGAC-3'	Brasher et al. (1998)
		Vvh-R: 5'-TGATTCCAGTCGATGCGAATACG-3'	Yamamoto et al. (1990)
V. cholerae	ISR 16S-23S rRNA	prVC-F: 5'-TTAAGCSTTTTCRCTGAGAATG-3' prVCM-R: 5'-AGTCACTTAACCATACAACCCG-3'	Chun et al. (1999)

529 [1]S: G or C ; R: A or G

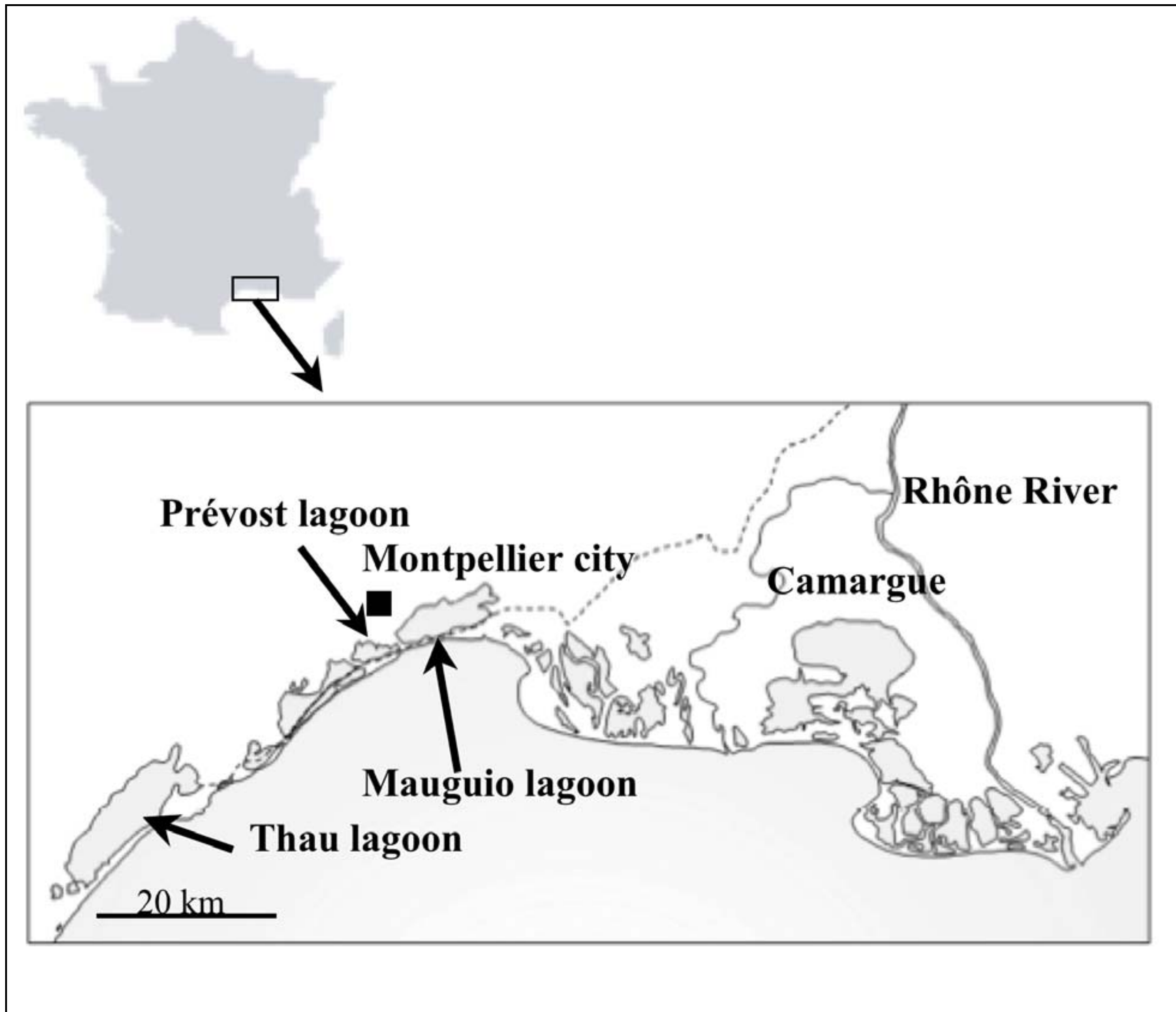
530

531 Table 2: Concentration (MPN/g of shellfish tissue) of *V. parahaemolyticus* (total, and
532 enteropathogenic, *trh2* and *tdh*), *V. vulnificus,* and *V. cholerae* in mussels and clams collected
533 in September, 2006, January and June, 2007 from Thau and Prévost lagoons.

		September 2006	January 2007	June 2007
Total *V. parahaemolyticus*	Thau lagoon clams	0.8 < 2.1 < 6.3	0.6 < 1.5 < 4.1	0.5 < 1.5 < 5
	Thau lagoon mussels	20 < 50 < 240	0	10 < 20 < 140
	Prévost lagoon mussels	3 < 9 < 39	0	80 < 210 < 640
V. parahaemolyticus trh2+	Thau lagoon clams	0	0	0
	Thau lagoon mussels	0	0	0.01<0.03<0.17
	Prévost lagoon mussels	3 < 9 < 39	0	0.02<0.07<0.28
V. parahaemolyticus tdh+	Thau lagoon clams	0.02<0.07<0.28	0.1<0.4<0.21	0.1<0.4<0.21
	Thau lagoon mussels	0.01<0.04<0.21	0	0
	Prévost lagoon mussels	0	0	0
V. vulnificus	Thau lagoon clams	0.01<0.04<0.21	0	6 < 15 < 41
	Thau lagoon mussels	0	0	0.01<0.04<0.21
	Prévost lagoon mussels	0	0	0
V. cholerae	Thau lagoon clams	0	0	0
	Thau lagoon mussels	0	0	0
	Prévost lagoon mussels	0	0	0

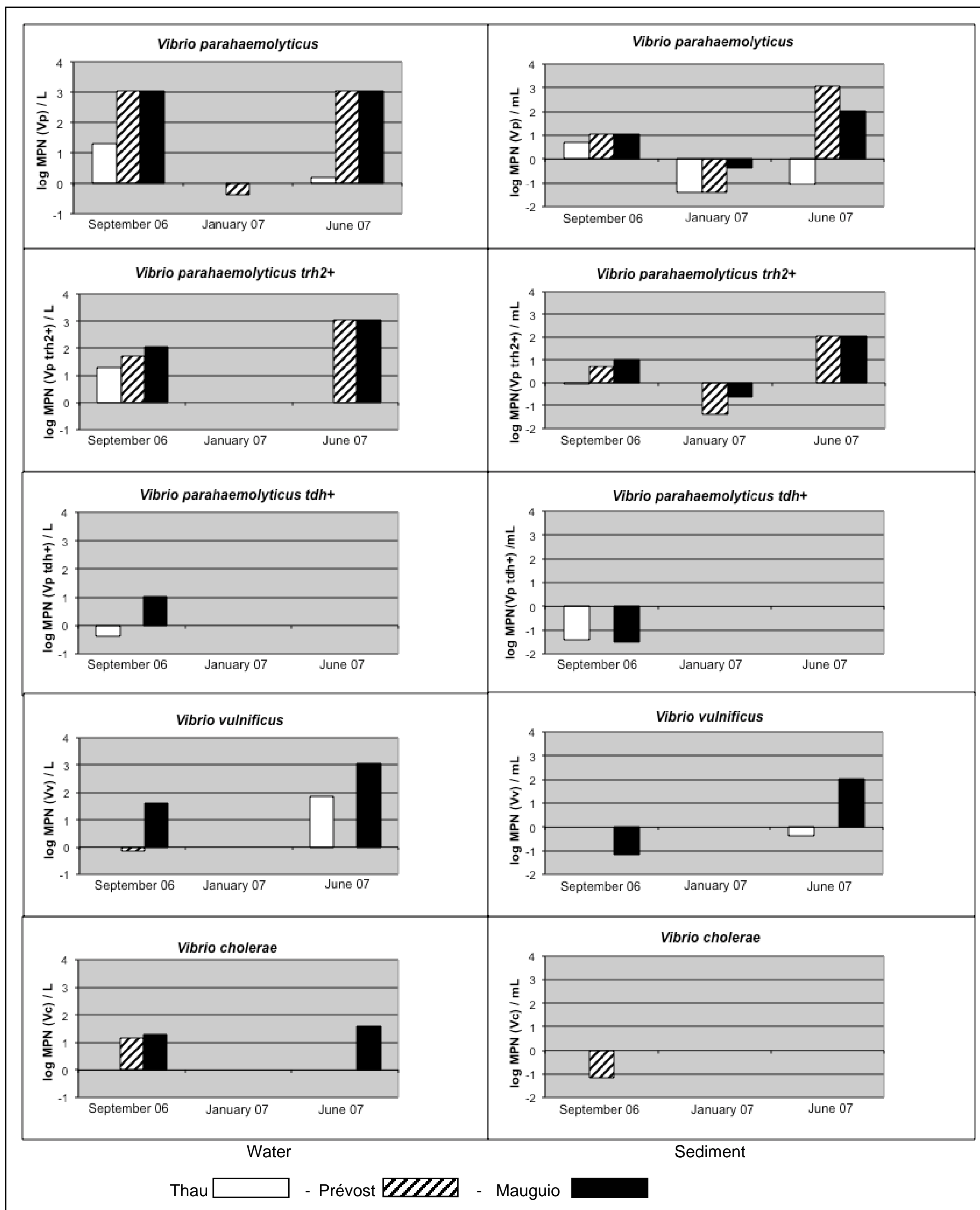
534

535 Figure 1



536 Figure 2

538



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