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# Infection dynamics of a *V. splendidus* strain pathogenic to *Mytilus edulis*: In vivo and in vitro interactions with hemocytes

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27 **Abstract**

28 The pathogenic strain *V. splendidus* 10/068 1T1 has previously been reported for its virulence  
29 to the blue mussel and for its capacity to alter immune responses. In this study, we expanded  
30 the knowledge on hemocyte-pathogen interactions by using *in vitro* and *in vivo* assays. *V.*  
31 *splendidus* 10/068 1T1 severely inhibited cell adhesion and acidic vacuole formation unlike the  
32 innocuous phylogenetically related *V. splendidus* 12/056 M24T1 which had no effect on these  
33 cell functions. Furthermore, the virulent bacteria decreased hemocyte viability (59% of viability  
34 after 24h). Infection dynamics were explored by using a model based on water tank cohabitation  
35 with septic mussels infected by GFP-tagged *V. splendidus* 10/068 1T1. Experimental infections  
36 were successfully produced (16.6% and 45% mortalities in 3 days and 6 days). The amount of  
37 GFP *Vibrio* in seawater decreased during the experiment suggesting its horizontal transfer from  
38 diseased animals to healthy ones. At the same time periods, bacteria were detected in hemocytes  
39 and in various organs and caused necrosis especially in gills. Total hemocyte count and viability  
40 were affected. Taken together, our results indicate that the pathogen *V. splendidus* 10/068 1T1  
41 colonizes its host both by bypassing external defense barriers and impairing hemocyte defense  
42 activities.

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44 **Keywords:** innate immunity, bivalves, *Vibrio*, infection, experimental model, hemocyte

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## 56 **1. Introduction**

57 Heterotrophic bacteria belonging to the genus *Vibrio* are highly abundant in the aquatic  
58 environment, mostly in seawater [1]. These ubiquitous microorganisms persist in a variety of  
59 geographic areas in interaction with eukaryotic marine hosts including zooplankton, sponges,  
60 corals and molluscs [1]. *Vibrio* sp. show a remarkable biodiversity. Until now, more than 110  
61 species of *Vibrios* have been identified, displaying a variety of host association modalities that  
62 extend from symbiosis to virulent pathogenicity [2,3].

63 Many species of pathogenic *Vibrios* are known to be responsible for diseases in terrestrial or  
64 marine vertebrates and invertebrates. *Vibrio cholerae*, *Vibrio vulnificus* and *Vibrio*  
65 *parahaemolyticus* in particular cause severe disorders in humans [4,5]. In numerous aquatic  
66 organisms including fish [6], corals [7], shrimp [8] and shellfish [9], some *Vibrios* have been  
67 associated with serious infections. Because of the high economic loss generated in the  
68 aquaculture sector, many studies are dedicated to bacterial diseases, particularly in farmed  
69 bivalves [3,9,10]. Among the etiological agents, bacteria from the clade *Splendidus* have been  
70 repeatedly described in relation to mortality events. This polyphyletic group include 16 species  
71 with contrasted pathotypes [11,12]. Different strains have been implicated in mortalities of  
72 various bivalves, e.g. the Pacific oyster *Crassostrea gigas* [13–17], the Atlantic scallop *Pecten*  
73 *maximus* [18,19], the carpet shell clam *Ruditapes philippinarum* [20], the greenshell mussel  
74 *Perna canaliculus* [21] and recently the blue mussel *Mytilus edulis* [11].

75 While infection mechanisms are well studied in human invaders, little is known in the specific  
76 case of invertebrate pathogens. Some results have been gathered in different species, especially  
77 concerning host-pathogen interactions [11, 22–24]. However, invasion processes remain poorly  
78 documented. Understanding infection dynamics is an essential step for developing diseases  
79 management strategies [25]. In particular, there is a need for robust and standardized  
80 experimental models of *Vibrio*-bivalve interactions.

81 In a previous work, from mortality events reported by mussel farmers, we have isolated a *Vibrio*  
82 strain virulent to the blue mussel. *V. splendidus* 10/068 1T1 has been shown to alter hemocyte  
83 phagocytosis capacities, a key parameter of the immune defense system in mussels.  
84 Furthermore, a stable GFP-tagged *Vibrio* strain was constructed to facilitate the study of  
85 interactions between the microorganism and immune cells [11]. Fluorescent proteins (FP)-  
86 tagged microorganisms constitute a useful tool to monitor colonization processes. They have  
87 been used to elucidate early invasion events in squids [26,27] and bacterial dynamics in filter  
88 feeding oysters [28].

89 In the present study, we have investigated infection dynamics of *V. splendidus* 10/068 1T1 in  
90 *Mytilus edulis*. We describe (i) the development of an experimental infection model by water  
91 tank cohabitation with septic mussels, (ii) the localization of GFP bacteria in infected animals  
92 with the corresponding tissue lesions and (iii) *in vitro/in vivo* interactions between the  
93 pathogenic *Vibrio* strain and *Mytilus edulis* hemocytes.

## 94 **2. Material and methods**

### 95 **2.1. Mussel collection**

96 Adult mussels, *M. edulis* with shell length ranging from 4 to 5 cm, were collected on the  
97 intertidal rocky shore of Yport (0°18'52"E:49°44'30"N, France) between December 2015 and  
98 March 2016, immediately transported to the laboratory and placed in a temperature-controlled  
99 (10°C) aerated Biotop Nano Cube 60 seawater tank (Sera, Heinsberg, Germany) , equipped  
100 with mechanical and activated biological filtering. The animals were fed with algae (*Isochrysis*  
101 *galbana*) and maintained in these conditions for at least one week before use.

### 102 **2.2. Bacterial strains and culture conditions**

103 Two parental and GFP-tagged *V. splendidus*-related strains were used in this study: a virulent  
104 *V. splendidus* 10/068 1T1 isolated from mussel mortality events reported by professional  
105 (French national surveillance network REPAMO) in 2010 and an innocuous *V. splendidus*  
106 12/056 M24T1 isolated from mussel microflora in absence of mortality in the context of  
107 Bivalife European project in 2012 [11]. Bacteria were routinely cultivated overnight in LBS  
108 [Luria Bertani complemented with salt, NaCl 20 g.L<sup>-1</sup> (f.c.)] at 22°C. Stock cultures were stored  
109 at -80°C in LBS with glycerol 15% (v/v) supplemented with kanamycin 100 µg.L<sup>-1</sup> for GFP-  
110 tagged strains.

### 111 **2.3. In vitro hemocyte challenge**

#### 112 **2.3.1. Hemolymph collection**

113 Hemolymph was withdrawn from the posterior adductor muscle sinus, by gentle aspiration with  
114 a 1 mL syringe equipped with a 22G needle. Quality of samples was systematically checked by  
115 microscopic observation before using in bioassays. Samples containing protozoa, tissue  
116 fragments, low number of hemocytes were discarded.

#### 117 **2.3.2. Hemocyte adhesion**

118 Cells were incubated with bacteria at a ratio of 10 bacteria/hemocyte, or with sterile  
119 physiological water (NaCl 9 g.L<sup>-1</sup>), in a 24-well tissue-culture plates (Greiner). After 2, 4 and

120 6 hours at 15°C, the number of non adherent cells in the supernatant was counted by flow  
121 cytometer.

### 122 **2.3.3. Acidic vacuole formation**

123 Crude hemolymph was placed into individual wells of 24-well tissue-culture plates (Greiner)  
124 for cytometry or in 35 mm  $\mu$ -Dish (Ibidi) for microscopy. Cells were exposed to *Vibrio* strains  
125 at 10:1 ratio (bacteria:hemocytes) for 2 hours at 15°C.

126 LysoTracker (LysoTracker® Green DND-26, life technologies) at 2  $\mu$ M was added and cells  
127 were incubated for 30 minutes at 15°C in the dark. Hemocyte fluorescence was quantified by  
128 flow cytometry. For microscopy imaging, cells were washed with the marine physiological  
129 saline solution [MPSS (470 mM NaCl, 10 mM KCl, 10 mM CaCl<sub>2</sub>, 10 mM HEPES, 48,7 mM  
130 MgSO<sub>4</sub>), pH 7.8, 0.2  $\mu$ m filtered]. Hemocyte nuclei were counterstained with hoechst 33342 (5  
131  $\mu$ M, 15 min) and imaged by epifluorescence microscopy.

### 132 **2.3.4. Hemocyte viability**

133 Hemocytes were exposed to bacteria (10/068 1T1 and 12/056 M24T1) at 10<sup>8</sup> CFU.mL<sup>-1</sup> for  
134 different time periods (2, 4, 6, 18 and 24h) at 15°C. At each time point, propidium iodide was  
135 added (20  $\mu$ M) and cell viability was measured by flow cytometry.

## 136 **2.4. *In vivo* challenge**

### 137 **2.4.1. Mussel infection by water tank cohabitation model**

138 Bacteria were prepared at OD<sub>600nm</sub> of 1 as described in Ben Cheikh et al. [11]. Animals were  
139 anesthetized for 2–3 h at 16°C in a magnesium chloride solution (50 g.L<sup>-1</sup>, 1/4: v/v  
140 seawater/freshwater) under aeration. Subsequently, a volume of 100  $\mu$ L of bacterial suspension  
141 (2.10<sup>8</sup> CFU.mL<sup>-1</sup>) or filtered sterile seawater (FSSW) for the negative control was injected into  
142 the posterior adductor muscle. After injection, the animals were transferred to tanks (3 replicate  
143 tanks, 10 mussels per tank) filled with 2L of UV-treated and filtered seawater supplemented  
144 with 50 mL of phytoplankton (*Isochrysis galbana*). After 24 hours, moribund animals were  
145 sacrificed by severing their adductor muscle and placed in cohabitation with a group of 10  
146 apparently healthy mussels. For the negative control, mussels injected with FSSW (alive) were  
147 sacrificed and used in the same conditions. After 72 hours of cohabitation, injected mussels  
148 were removed. During the experiment, animals were maintained under static conditions at 16°C  
149 with aeration. Mortality was monitored each day over a six days period. Animals were  
150 considered to be dead when the valves did not close following stimulation. Newly dead mussels  
151 were removed from the tanks.

#### 152 **2.4.2. Bacteria counting in seawater**

153 Seawater was sampled from cohabitation tanks each day during the experiment period. 100  $\mu$ L  
154 of samples serially diluted in sterile physiological water (NaCl 9 g.L<sup>-1</sup>) was plated on LBS agar  
155 supplemented with kanamycin 100  $\mu$ g.L<sup>-1</sup>. After 48h at 22°C, colonies were counted. The  
156 presence of GFP colonies was verified under a fluorescence stereo microscope (Leica  
157 microsystems).

#### 158 **2.4.3. Hemocyte cellular parameters analysis**

159 Hemolymph was sampled from exposed mussels and mixed with cold Alsever's solution (300  
160 mM NaCl, 100 mM Glucose, 30 mM sodium Citrate, 26 mM citric acid, 10 mM EDTA, pH  
161 5.4) for cytometry analysis.

162 Variation in hemocyte count and the percentage of cells containing GFP-tagged bacteria was  
163 determined. Cell viability was investigated by adding propidium iodide (20  $\mu$ M).

#### 164 **2.4.4. Histological and Immunohistochemical analyses**

165 During cohabitation infection, mussels were sampled at different time periods and removed  
166 from their shell. Tissues were fixed in Davidson's solution (20% formaldehyde 36%, 30%  
167 FSSW, 10% glycerol, 30% ethanol 95% and 10% acetic acid) for 48h, dehydrated in a graded  
168 series of ethanol, and embedded in paraffin. Consecutive 3-5  $\mu$ m thick sections were adhered  
169 to Superfrost [hematoxylin-eosin (HE) staining] or Superfrost Plus [immunohistochemistry  
170 (IHC)] microscope slides.

171 For tissue examination, sections were stained by classical hematoxylin-eosin protocol. Presence  
172 of listed pathogens (*Bonamia* sp., *Marteilia* sp., *Perkinsus* sp. and *Mikrocytos* sp.) was  
173 examined as well as trematods, copepods, *Mytilicola*, ciliates and gregarines. For lesions, we  
174 noticed the presence of necrosis, bacterial "foyer", hemocytes infiltration and granulomas into  
175 the different tissues: gills, gonads, digestive glands, mantle, muscle, kidney and digestive tube.  
176 The lesions in each organ were coded as follows: 0 (absence of lesion); 1 (low), 2 (moderate),  
177 3 (high). "Low" corresponds to at least one observation in 20 fields of the slide, "moderate"  
178 corresponds to at least 5 observations in 10 fields of the slide and "high" corresponds to at least  
179 10 observations in 10 fields of the slide.

180 For IHC, a polyclonal antibody raised in rabbit against recombinant full length GFP protein  
181 was used (Ab290, Abcam). The specificity of this antiserum has been previously tested and  
182 optimized. Immuno-labellings were performed with a Benchmark automate (Ventana-Roche)  
183 by Histalim (Montpellier). Sections were incubated for 32 min with primary antibody (1:5000)  
184 and revealed with Ultraview Red Alkaline Phosphatase kit (Roche Diagnostics). Slides were

185 finally numerized with a Nanozoomer x20 (Hamamatsu). Labeling of bacterial-like cells was  
186 coded following the same criteria used for lesion observations: 0 (absence of labelling); 1 (low),  
187 2 (moderate), 3 (high).

188

## 189 **2.5. Statistical analyses**

190 Statistical analysis was performed by using SigmaPlot 12 (Systat Software Inc., Chicago, IL).  
191 Replicates were averaged and the values were tested for normality (Shapiro-Wilk) and paired  
192 comparisons were performed by Student's t-tests or by Mann-Whitney rank sum tests in case of  
193 unequal variance. Statistical significance was accepted for \* $p < 0.05$ , \*\* $p < 0.01$  or \*\*\* $p <$   
194  $0.001$ .

## 195 **3. Results**

### 196 **3.1. Hemocyte adhesion**

197 The effect of *V. splendidus*-related strains on hemocyte-substrate adhesion was evaluated *in*  
198 *vitro* for different time of incubation (**Figure 1**). Hemocyte attachment to the culture dish was  
199 significantly affected after 2h of exposure to the virulent *V. splendidus* 10/068 1T1. Moreover,  
200 the number of detached hemocytes increased for 6h incubations. In contrast, when exposed to  
201 the innocuous *Vibrio* strain 12/056 M24T1, non-adherent cells increased slightly after 2h  
202 exposure and then decreased.

### 203 **3.2. Acidic vacuole formation**

204 The capacity of *M. edulis* hemocytes to generate phagolysosome proliferation after exposure to  
205 virulent and non-virulent bacteria *in vitro* was investigated by flow cytometry and  
206 epifluorescence microscopy (**Figure 2**). The lysotracker signal, indicative of acidic vacuole  
207 formation, was significantly higher in hemocytes challenged with the strain 12/056 M24T1 than  
208 in cells co-cultured with the strain 10/068 1T1 ( $p < 0.001$ , **Figure 2a**). Moreover, in the case of  
209 exposure to 12/056 M24T1, numerous phagolysosomic compartments surrounding the nucleus  
210 can be found (**Figure 2b**). Microscopic observation also confirmed the absence of large acidic  
211 compartments in cells challenged with the virulent strain.

### 212 **3.3. Hemocyte viability**

213 Hemocyte viability was monitored after exposure to *V. splendidus*- related strains for different  
214 time durations. Cell viability was stable during the first 6 hours for both strains with values  
215 corresponding to 92% of viable cells (**Figure 3**). Then, hemocyte viability slightly decreased  
216 until 24h post exposure to the innocuous strain 12/056 M24T1, but was never lower than 80 %.



217 In contrast, for the same time periods, the viability of cells incubated with the strain 10/068 1T1  
218 was significantly affected, reaching a percentage of viable cells of only 59% after 24h exposure  
219 to the virulent strain.

#### 220 **3.4. *Vibrio* infection by cohabitation challenge**

221 Experimental infection of mussels with the pathogenic *V. splendidus* 10/068 1T1 was obtained  
222 by water tank cohabitation assays. First mortalities appeared after 3 days (16.6%) and increased  
223 progressively to reach almost 45% at the 6<sup>th</sup> day (**Figure 4**). No mortality was observed in  
224 control tanks.

#### 225 **3.5. Seawater bacteria count during infection**

226 Bacteria seawater density in cohabitation tanks was monitored during infection (**Figure 5**).  
227 After 48h of cohabitation, the *Vibrio* density reached  $3.10^6$  CFU/mL, then decreased  
228 progressively to  $2.10^3$  CFU/mL after 5 days.

#### 229 **3.6. Hemocyte parameters analysis during the experimental infection**

230 Different hemocyte parameters were investigated during cohabitation assays with septic  
231 mussels infected by the virulent strain 10/068 1T1 (**Figure 6**). In hemolymph sampled from the  
232 adductor muscle, the total hemocyte count increased significantly 48h after the cohabitation  
233 onset and then decreased until the end of the exposure to reach control values (**Figure 6a**).  
234 Interestingly, GFP bacteria were detected in hemocytes. The percentage of cells containing  
235 GFP-tagged *Vibrio* was also higher at the beginning of the exposure and then decreased  
236 progressively (**Figure 6b**). Furthermore, hemocyte viability slightly decreased compared to the  
237 control but remained almost stable during *Vibrio* infection (80% of viable cells at day 2 and  
238 74% at day 5, **Figure 6c**).

#### 239 **3.7. Tissue infection by bacteria**

240 GFP-*Vibrios* were detected mainly in gills of 8 out of 31 live animals and 9 out of 9 moribund  
241 animals, either in the pallial cavity, forming aggregates, attached to apical cilia or between  
242 ordinary gill filaments (**Figure 7A, C**). Some labeling were also noticed in esophagi or stomach  
243 of 4 out of 28 live animals (**Figure 7D**). Main observed lesions correspond to necrosis of  
244 epithelia (gills and digestive tissues, respectively 30 and 8 out of 40 animals) and hemocyte  
245 infiltrations (17 out of 40 animals), as well as granulomas in the mantle (17 out of 40 animals)  
246 (**Figure 7B, supplementary figure 1C-E, Table 1**). Moribund animals contained bacteria in  
247 large amounts and presented general necrosis patterns even if some organs were obviously not  
248 affected (muscle, gonad, kidney and mantle) (**Figure 7B, Table 1**).

249 Some trematodes *metacerca* in 36 out of 40 animals, copepods in 3 out 40 animals, ciliates in  
250 28 out of 40 animals or gregarines in 7 out of 40 animals, were noticed (**supplementary figure**  
251 **1, Table 1**). However, the most noticeable microorganism present on hematoxylin-eosin  
252 strained slides corresponds to bacteria. Seventeen of the 40 analyzed individuals present  
253 bacterial cells into gills (8 low amount, 6 moderate and 3 high). Bacteria were also noticed in  
254 digestive tissues (10 individuals – 6 low and 4 moderate) and mantle (9 individuals – 3 low and  
255 6 moderate) (**supplementary figure 1D, Table 1**). No listed parasites were found in the 40  
256 mussels (19 females and 21 males).

## 257 **4. Discussion**

258 In a previous study, we have reported the virulence of *V. splendidus* 10/068 1T1 to the blue  
259 mussel and its capacity to alter the host immune response [11]. Herein, we expand the  
260 knowledge on mussel-pathogen interactions by exploring infection dynamics via water tank  
261 cohabitation experimental model and by studying hemocyte responses via *in vitro/in vivo*  
262 assays.

### 263 **4.1. In vitro hemocyte-pathogen interactions**

264 To elicit cell-mediated immune responses, adhesion phenomena are crucial. In our experiments,  
265 *V. splendidus* 10/068 1T1 severely altered hemocyte attachment to the culture substratum. After  
266 2 hours of incubation in the presence of the virulent *Vibrio*, the number of non-adherent cells  
267 was higher than in non-treated cultures or in cells co-incubated with the innocuous bacteria.  
268 This effect increased after 6h of exposure to *V. splendidus* 10/068 1T1. Similar observations  
269 were made for other *Vibrio* species with bivalve hemocytes. For example, *V. tasmaniensis*  
270 LGP32 decreased hemocyte adhesion capacity in *Mytilus edulis* [29], *Mytilus galloprovincialis*  
271 [30] and *Mya arenaria* [31]. The same response was reported for *Crassostrea gigas* hemocytes  
272 challenged with *V. aestuarianus* 01/32 [32] and cells of *Ruditapes philippinarum* in contact  
273 with *V. tapetis* [33]. Despite the robustness of these observations, specific mechanisms by  
274 which a pathogen induces hemocyte detachment from adhesion surfaces remain poorly  
275 understood. Some studies suggested the involvement of proteases secreted by the bacteria and  
276 responsible for disruption of cytoskeleton, cell adhesion molecules and extracellular matrix  
277 components. In the Pacific oyster, hemocyte treatment with extracellular products (ECPs) of *V.*  
278 *aestuarianus* 01/32 or *V. tubiashii* 07/118 T2 inhibited cell binding to culture dish [34,35].  
279 Furthermore, the hydrolytic action of extracellular effectors capable to degrade muscle  
280 collagen, bovine actin and fibronectin proteins has been shown [35]. In addition of abolishing  
281 hemocyte attachment to substrate, alteration of immunocyte adhesion capabilities by ECPs also

282 probably affects phagocyte activity. In this regard, we have previously shown that ECPs from  
283 *V. splendidus* 10/068 1T1 inhibit *M. edulis* hemocyte phagocytosis [11].

284 Upon entrance into hemocytes, the fate of bacteria depends on phagosome biogenesis and  
285 maturation. Intracellular trafficking and killing of engulfed microorganisms is a highly  
286 choreographed process driven by subsequent fusion and fission events during which the  
287 maturing phagosome acquires the characteristics of degradative acidic lysosomes [36,37]. In  
288 our study, *V. splendidus* 10/068 1T1 inhibited acidic vacuole formation in mussel hemocytes  
289 while the innocuous phylogenetically related to *V. splendidus* 12/056 M24T1 had no effect.  
290 Such mechanism has been described for intracellular pathogens as strategy to survive and  
291 maintain infection within cells. This is the case of *Legionella pneumophila*, the causative agent  
292 of Legionnaire's pneumonia, known for its ability to manipulate host cell vesicular trafficking  
293 pathways and to inhibit phagosome-lysosome fusion [38,39]. In the same way, *Mycobacterium*  
294 sp. reside inside vacuoles and arrest the fusion with late endosomal/lysosomal organelles [40,  
295 41]. For *Vibrio* species, few reports have shown their capacity to adopt intracellular stages [42–  
296 44]. To our knowledge, only *V. tasmaniensis* LGP32 has been described as a facultative  
297 intracellular pathogen of the Pacific oyster, able to modulate phagosome maturation as well as  
298 oxidative response [24]. More recently, Vanhove et al. [45] showed the intracellular surviving  
299 of this strain until hemocyte cytolysis (more than 25% of cell lysis after 17h incubation). In  
300 accordance with these findings, it seems that *V. splendidus* 10/068 1T1 displays a similar  
301 infection strategy to *V. tasmaniensis* LGP32. Besides altering phagosome maturation and ROS  
302 production [11], this *Mytilus edulis* pathogen affected hemocyte viability and induced almost  
303 39% of dead cells after 18h. The latter point prove the cytotoxicity of the bacteria and suggests  
304 its surviving into the cells.

#### 305 **4.2. *In vivo* infection dynamics**

306 Understanding pathogenesis processes requires an animal model of infection [46]. In our  
307 previous study, we have demonstrated the virulence of *V. splendidus* 10/068 1T1 towards the  
308 blue mussel by injection [11]. In spite its efficacy and repeatability, this method does not reflect  
309 the realistic way of host-bacteria interaction. Herein, we successfully reproduced experimental  
310 infection by cohabitation assays. In these experimental conditions, first mortalities appeared  
311 after 3 days and increased progressively suggesting a necessity of time for bacteria transfer  
312 from diseased animals and the occurrence of a latency period in healthy mussels. At the same  
313 time intervals, the amount of GFP-*Vibrio* released in the seawater decreased ( $3 \cdot 10^6$  CFU/mL at  
314 the 2<sup>nd</sup> day to  $2 \cdot 10^3$  CFU/mL at the 6<sup>th</sup> day). In addition to probable adhesion/penetration into

315 its host, this decline could result from bacteria mortality or adhesion to tank walls. Though,  
316 *Vibrio* were also detected in healthy mussel tissues confirming a transmission of a part of them  
317 from infected animals during the cohabitation period.

318 Up to now, only few works have investigated non-invasive methods for the study of infectious  
319 diseases in bivalves and they were limited to Pacific oysters. In this model, successful  
320 experimental vibriosis were induced by cohabitation [47,48] or by immersion assays [49]. In  
321 our case, unlike cohabitation experiments, the bathing procedure did not cause any mortality  
322 (data not shown). This negative result reveals the complexity of infection process. Bacteria may  
323 require a priming infectious niche or a cooperation with mussel microflora to initiate some  
324 virulence mechanisms [50].

325 During experimental infection, the number of circulating cells temporary increased at day 2  
326 post-challenge and then stabilized to a value not distinct from control. Similar transient  
327 hemocytosis have been described in diverse bivalve species like *M. edulis* [51], *M.*  
328 *galloprovincialis* [52] and *R. philippinarum* [53] in response to physical stresses or pathogen  
329 threats. Such variation in circulating hemocytes have been explained by cell proliferation [51].  
330 Mobilizations of peripheral hemocytes from tissues consecutively to bacterial infection were  
331 also evoked to account for by a transient increase of circulating cells [32,53]. Inversely, back  
332 infiltration of infected sites by hemocytes could explain a consecutive decrease of total  
333 hemocyte count in hemolymph. Alternatively, the decrease of cell concentration observed  
334 secondarily to hemocytosis can also originate from a *Vibrio* induced hemocyte death. The  
335 hypothesis get confirmation from our experiments showing a loss of viability both for  
336 hemocytes exposed to *Vibrio in vitro* and for hemocytes withdraw from infected animals. It is  
337 also in good agreement with mortalities observed after 3 days of cohabitation with septic  
338 mussels. Furthermore, *V. splendidus* 10/068 1T1 was detected in hemocytes and the population  
339 of bacteria-containing hemocyte declined progressively with time. The latter result is probably  
340 coherent with a pathogenicity involving a phagocyte lysis and a bacteria release.

341 In addition to hemocyte parameters analysis, histology was performed on infected mussels.  
342 Many organisms were identified in tissues of living and moribund mussel (trematodes,  
343 copepods, *Ciliophora*, ciliates and gregarines). The presence of parasites is not surprising since  
344 animals were collected from natural areas. Their potential interaction with pathogens are still  
345 unknown. GFP-*Vibrio* were localized in diverse organs and obviously caused necrosis of  
346 digestive gland and gills principally. Data concerning bacterial diseases in bivalves indicate that  
347 infections are initiated at mucosal interfaces [54]. Nevertheless, in order to establish a

348 chronology, the entry route of bacteria has to be identified. Few studies explored portal access  
349 of pathogens in bivalves. For example, the organic matrix of the shell has been described as a  
350 putative entryway of the pathogenic *V. tapetis* in the clam *R. philippinarum* [55,56]. More  
351 recently, a study suggested the involvement of hemocytes in microbe transport because of their  
352 ability to migrate across mucosal epithelia and to translocate within hours from pallial surfaces  
353 to underlying tissues and to the circulatory system [54]. This hypothesis may be plausible in  
354 our study considering the cytotoxicity of *V. splendidus* 10/068 1T1 to hemocytes. Further  
355 investigations at the first hours of infection are needed to elucidate whether hemocytes are the  
356 first or the last target of pathogens.

## 357 **5. Conclusion**

358 *V. splendidus* 10/068 1T1 has been previously reported to be pathogenic to the blue mussel. In  
359 this study, we first investigated hemocyte-bacteria interactions. *In vitro* challenges demonstrate  
360 the capacity of *Vibrio* strain to alter the maturation of the phagolysosome, the cell adhesion and  
361 viability. Then, we successfully established a non-invasive experimental infection model via  
362 cohabitation. Bacteria were able to bypass defense barriers and were detected in diverse organs  
363 and in hemocytes. The number and the viability of circulating hemocytes were altered. Taken  
364 together, our results confirm the virulence of *V. splendidus* 10/068 1T1 towards *M. edulis*  
365 hemocytes and suggest the use of immune cells as pathogen vehicles to spread the infection in  
366 the whole organism. Furthermore, besides the description of an original infection process, this  
367 study can be useful to be compared with natural mortalities occurred in the field.

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569 **Figure legend**

570 **Figure 1.** Effect of *V. splendidus*-related strains on hemocyte adhesion *in vitro*.  
571 The number of non-adherent cells was evaluated after exposure to the virulent *V. splendidus*  
572 10/068 1T1 and to the non-virulent *V. splendidus* 12/056 M24T1 for 2, 4 and 6h. Data are  
573 expressed as mean  $\pm$  SEM (n=5), \* indicates values significantly different from control and §  
574 marks results that significantly differ from values obtained with the non-virulent bacteria  
575 12/056 M24T1 (p<0.05, Student's t-test)

576 **Figure 2.** Acidic vacuole formation in hemocytes after exposure to *V. splendidus*-related  
577 strains. (a) Flow cytometry analysis of hemocytes exposed *in vitro* to virulent or non-virulent  
578 bacteria during 2h and incubated 30 min with lysotracker at 0.4  $\mu$ M. Data are expressed as mean  
579 of fluorescence  $\pm$  SEM, arbitrary units (A.U.), n=5. \*\*\* indicates values significantly different  
580 from control (Student's t-test, p<0.001). (b) Fluorescence microscopy of hemocytes exposed *in*  
581 *vitro* to virulent or non-virulent bacteria during 2h and incubated with lysotracker green (0.4  
582  $\mu$ M, 30 min) and Hoechst 33342 (5  $\mu$ M, 15 min).

583 **Figure 3.** Effect of exposure to bacteria on hemocyte viability *in vitro*. Hemocytes were  
584 incubated with *V. splendidus*-related strains 10/068 1T1 or 12/056 M24T1 for different time  
585 durations. Viability was determined by flow cytometry after propidium iodide staining. Data  
586 are expressed as mean  $\pm$  SEM, n=4. \*\* indicates values significantly different from the control  
587 p<0.01 Student's t-test).

588 **Figure 4.** Cumulative mortalities recorded after experimental infections of adult mussels by  
589 water tank cohabitation with septic mussels. GFP-tagged *V. splendidus* 10/068 1T1 strain was  
590 injected intramuscularly to mussels. 24h post injection, moribund animals were sacrificed and  
591 placed in cohabitation with healthy mussels for 72h and then removed. Cohabitation assays  
592 with mussels injected with FSSW were used as control. Data are mean  $\pm$  SEM of cumulative  
593 mortalities in triplicate tanks.

594 **Figure 5.** *V. splendidus* 10/068 1T1 count in water tank seawater during experimental infections  
595 *in vivo* by cohabitation with septic mussels. Seawater was sampled daily during cohabitation  
596 assays and plated on LBS kanamycin agar plates. Bacteria concentration was determined over  
597 time (CFU/mL, mean  $\pm$  SEM, n=3)

598 **Figure 6.** Analysis of hemocyte parameters during experimental infections by *V. splendidus*  
599 10/068 1T1 via water tank cohabitation with septic mussels. Hemolymph was sampled over  
600 time from cohabited mussels and (a) absolute hemocyte concentration, (b) percentage of  
601 hemocyte containing GFP bacteria and (c) hemocyte viability were monitored by flow  
602 cytometry. Data are expressed as mean  $\pm$  SEM (n=4-13). Values significantly different from  
603 control are indicated (\* p<0.05, \*\* p<0.01\*, \*\*\*p<0.001, Student's t-test).

604 **Figure 7.** Histological observations of mussel tissues during experimental infections by *V.*  
605 *splendidus* 10/068 1T1 via water tank cohabitation with septic mussels. GFP-tagged bacteria  
606 were detected by immunohistochemistry (pink labeling). Tissues were counter-stained with  
607 hematoxylin. A-C: Gills, D: esophagi and stomach. Scale bars of 50 or 100  $\mu$ m are indicated.

608 **Supplementary figure 1.** Histological observations of hematoxylin-eosin stained sections of  
609 mussel tissues during experimental infections by water tank cohabitation with septic mussels.  
610 A. Unidentified trematode in digestive glands (metacercaria), B. *Mytilicola* sp. in digestive lumen,  
611 C. Unidentified copepod and hemocytes infiltration in gills, D. Bacteria, ciliates and gill  
612 necrosis, E. Digestive glands and esophagi necrosis, F. Inflammatory granulomas. Scale bars  
613 of 100  $\mu$ m, 250  $\mu$ m, 500  $\mu$ m or 1 mm are indicated.

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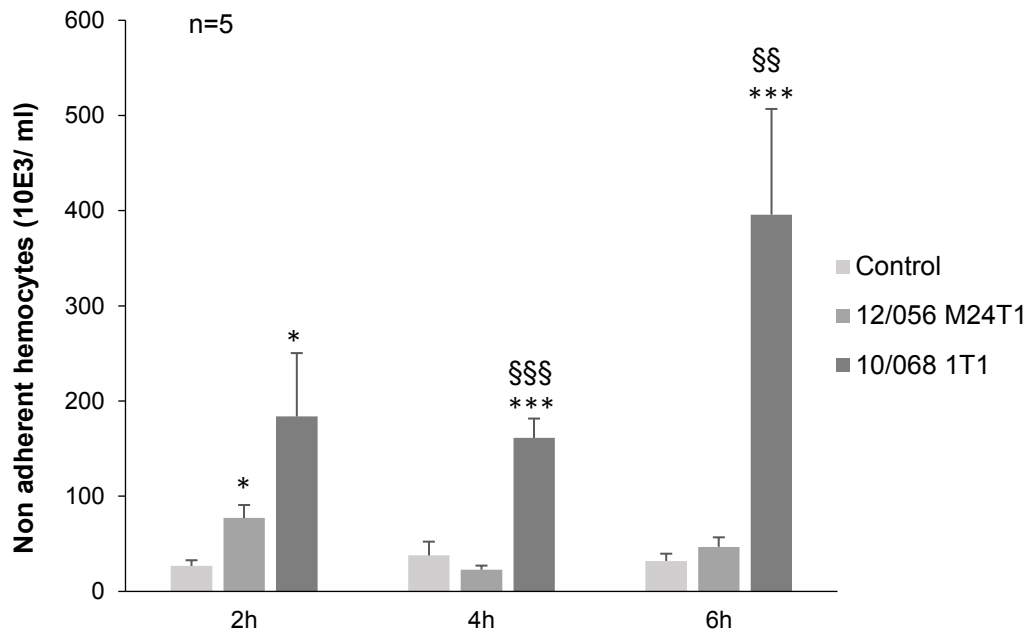
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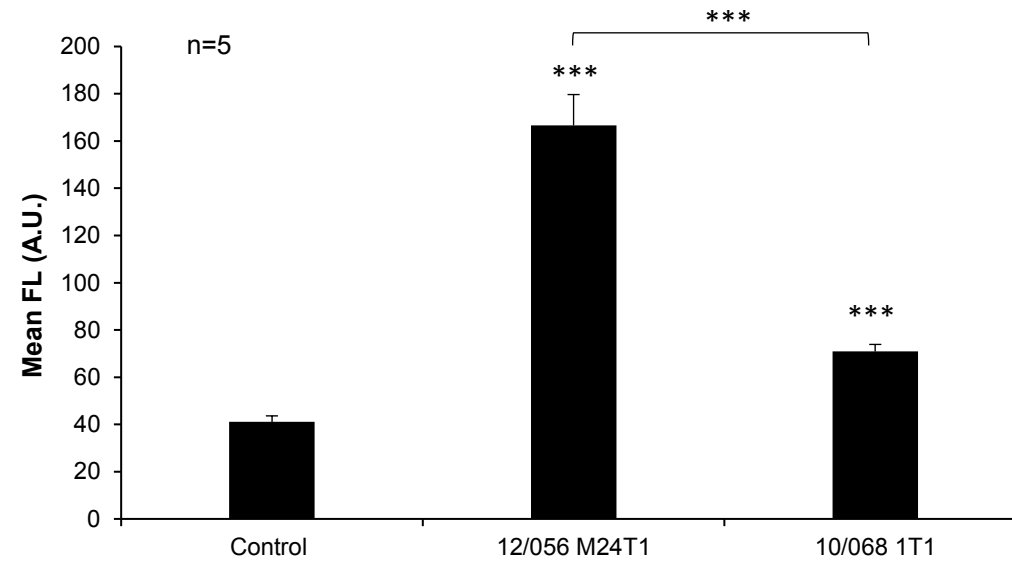
620 **Table 1:** Histological observations on hematoxylin-eosin stained sections of mussel tissues  
621 during experimental infections by water tank cohabitation with septic mussels. Presence of  
622 micro-organisms and lesions (as necrosis, infiltrations or granulomas) were noticed and  
623 classified in categories (0, 1, 2 or 3) as defined in material and methods section. A total of 40  
624 individuals were observed (21 males, 19 females; 38 live sacrificed animals, 9 moribunds).  
625 Number of observations is indicated, as well as the number of included moribunds in this  
626 count (includ. xM = including x moribunds).

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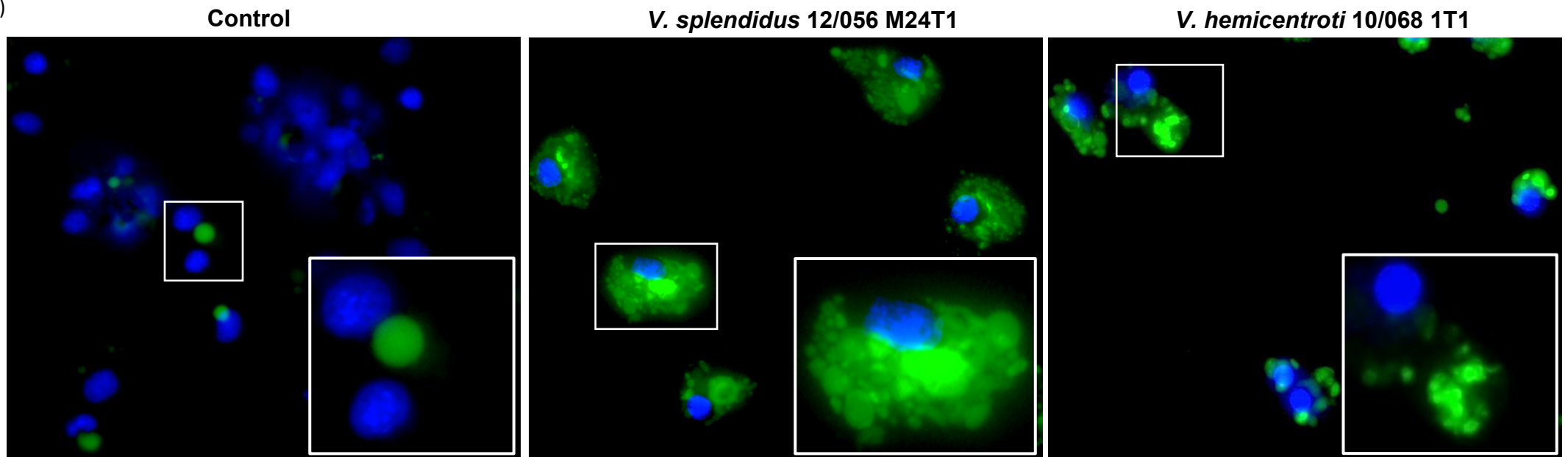
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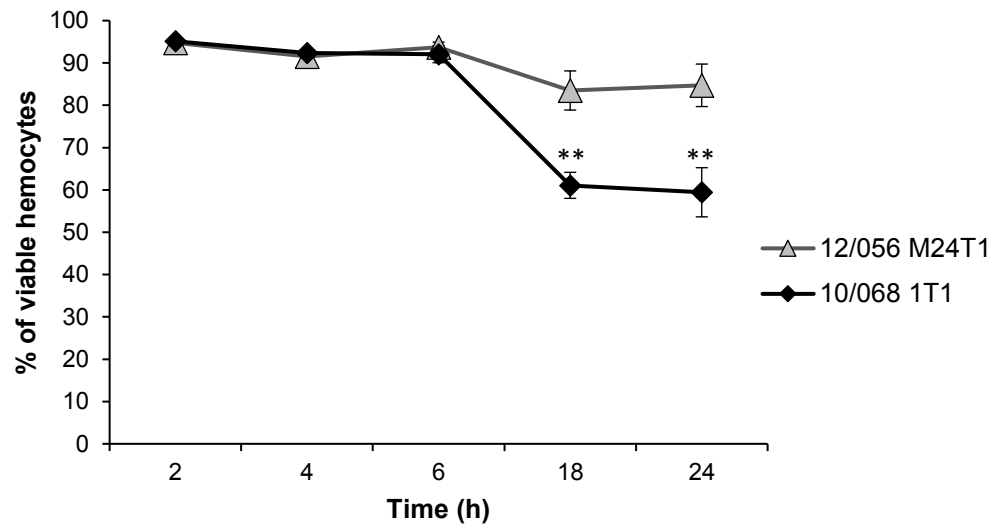


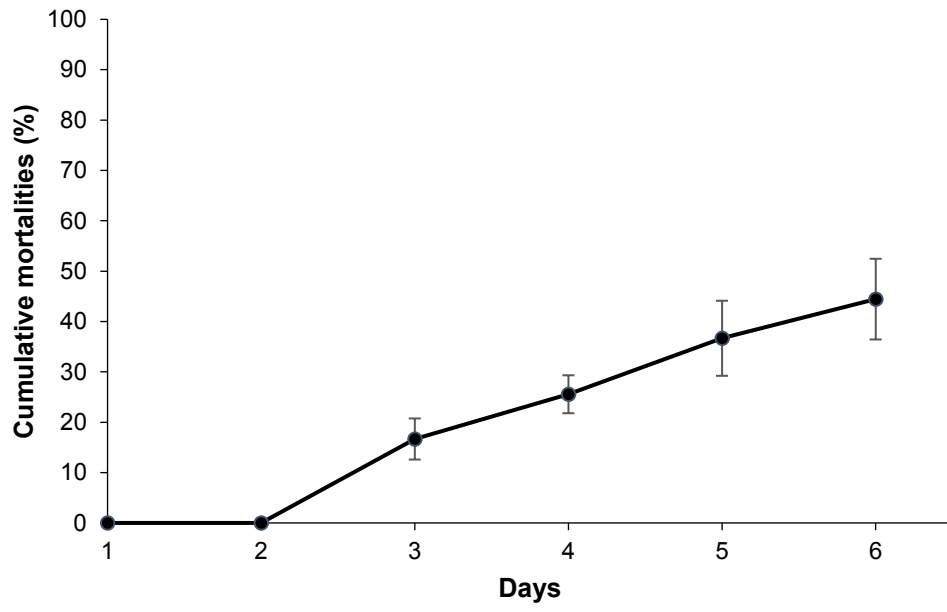
(a)



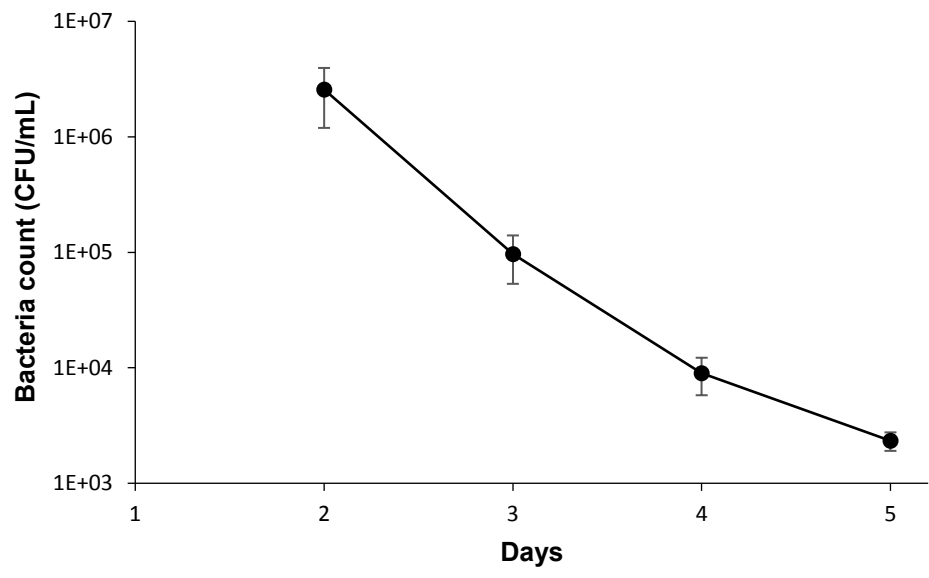
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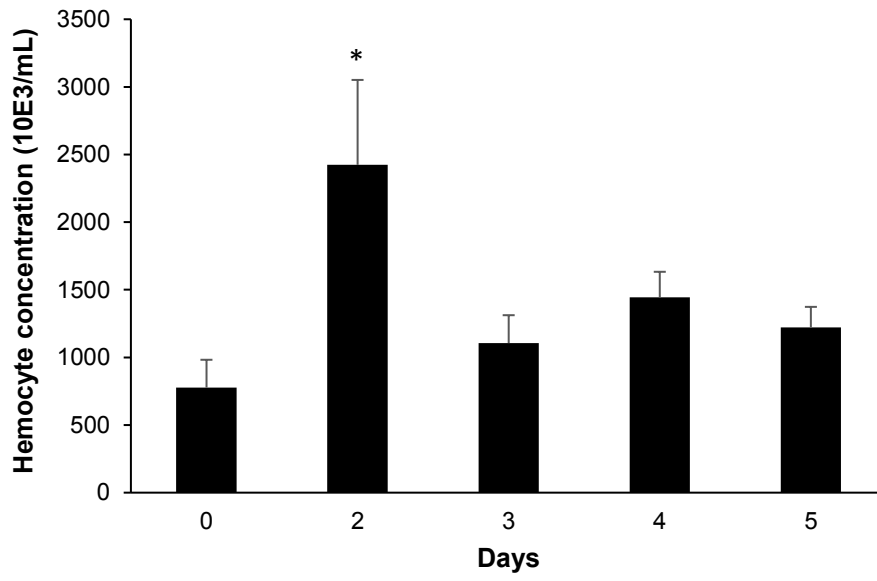




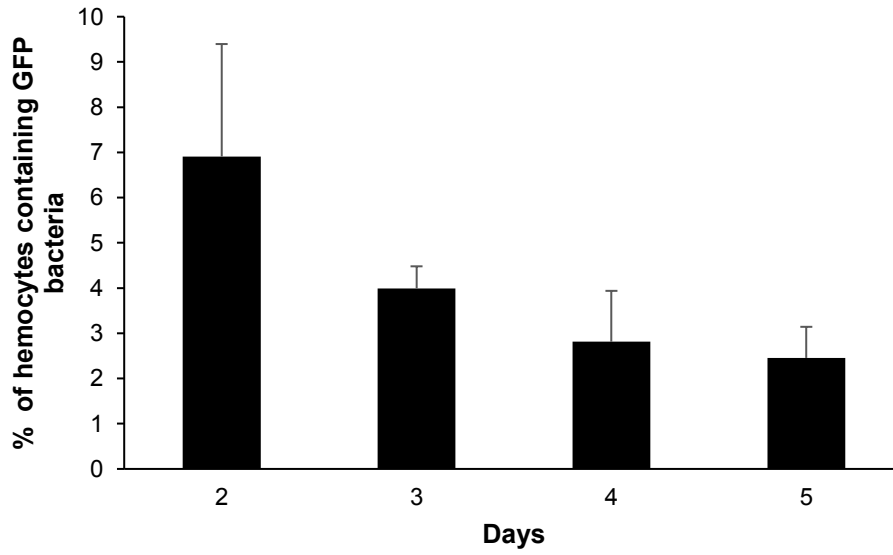




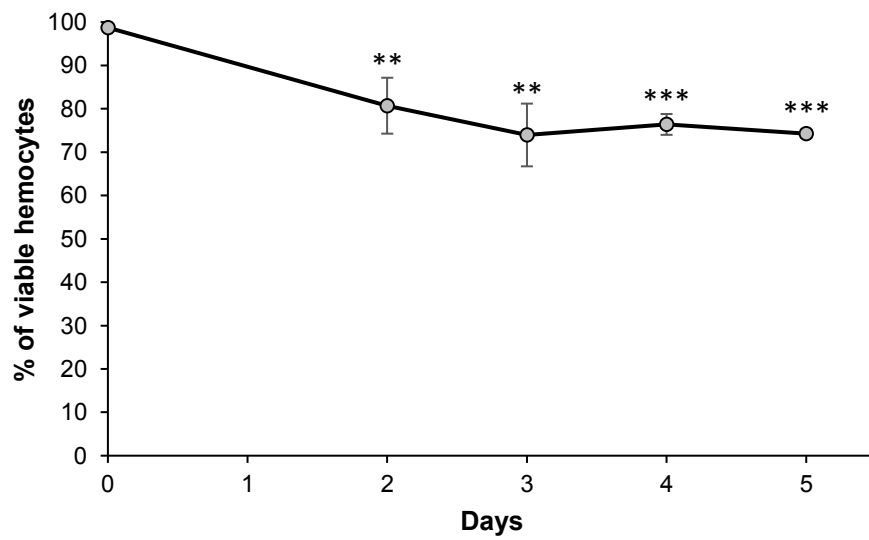
(a)

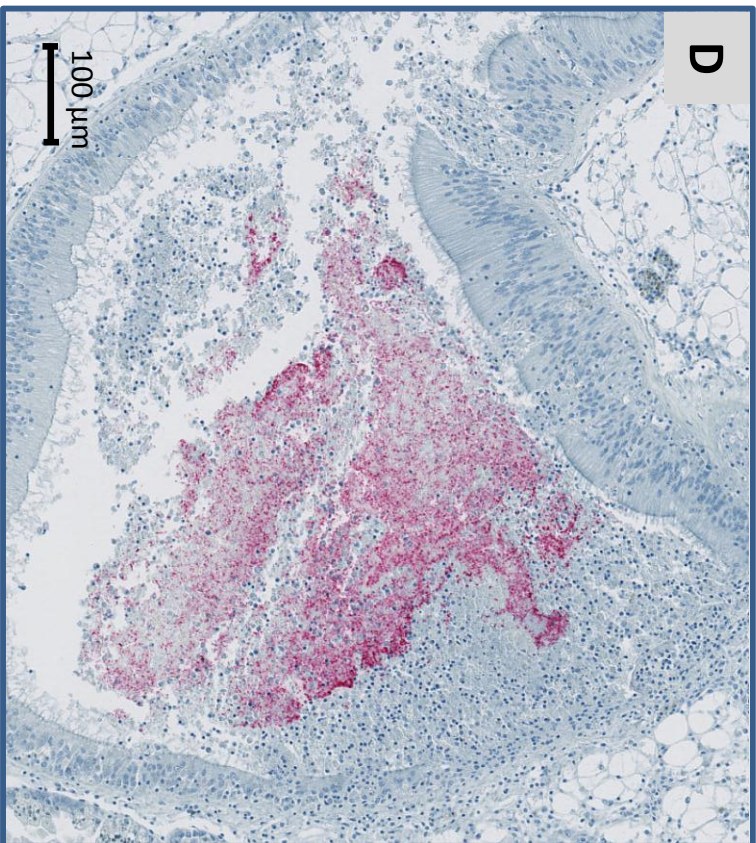
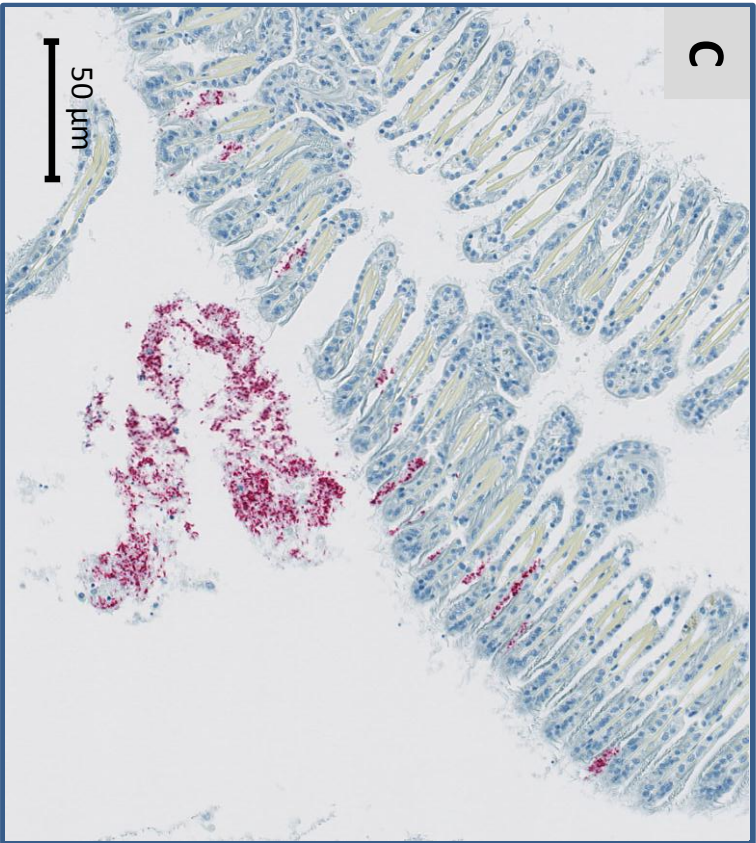
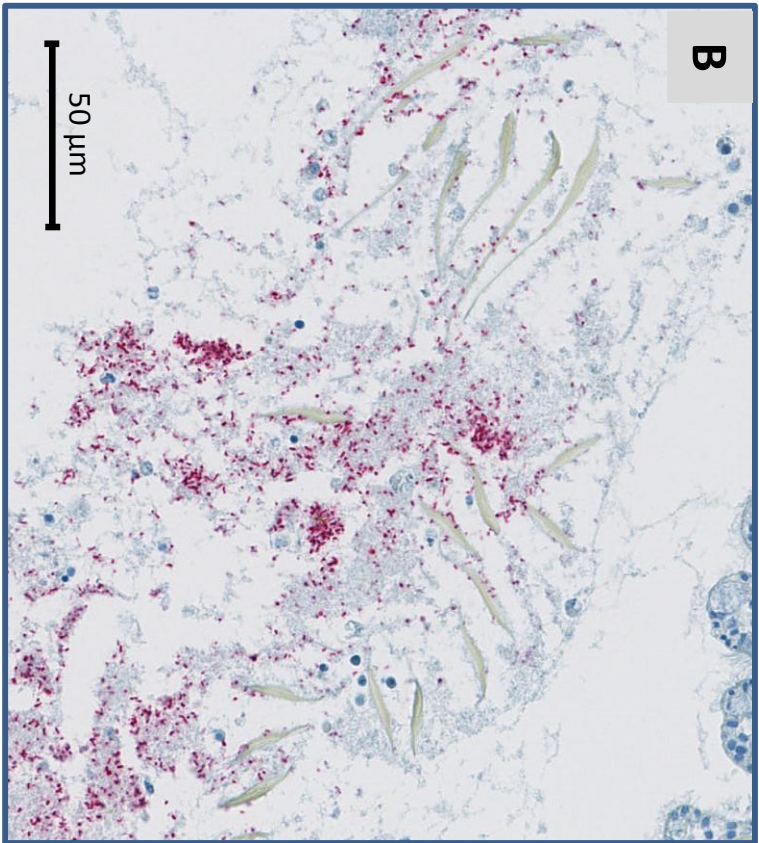
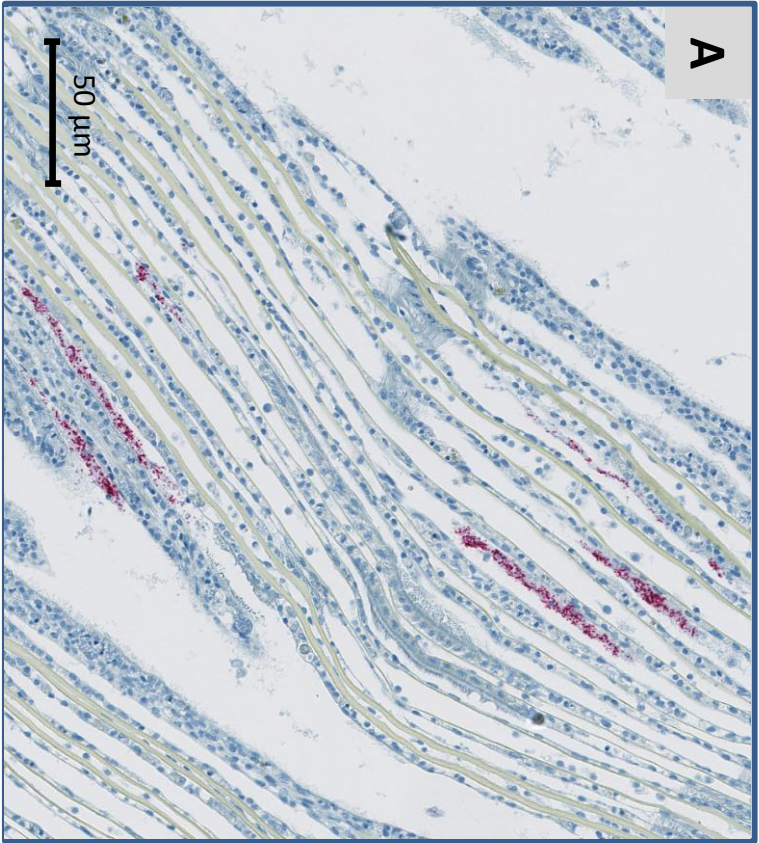


(b)

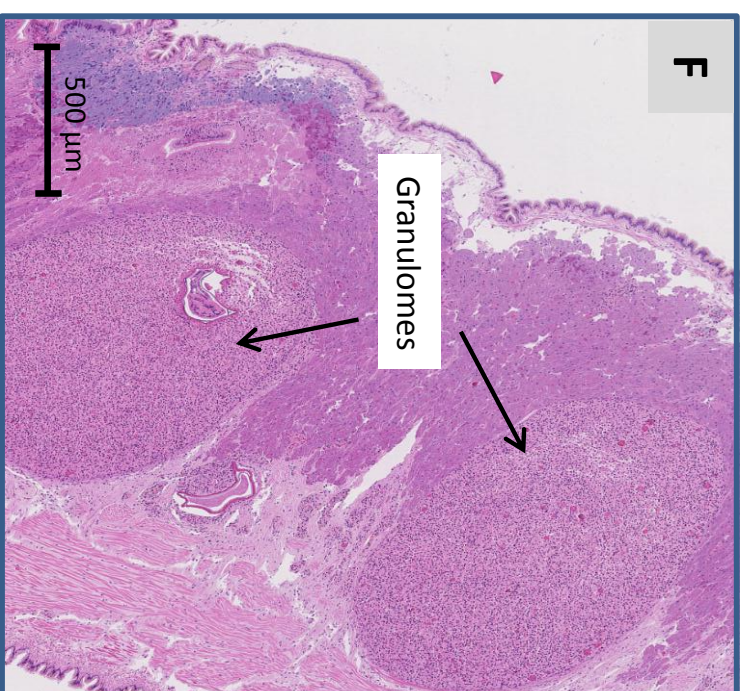
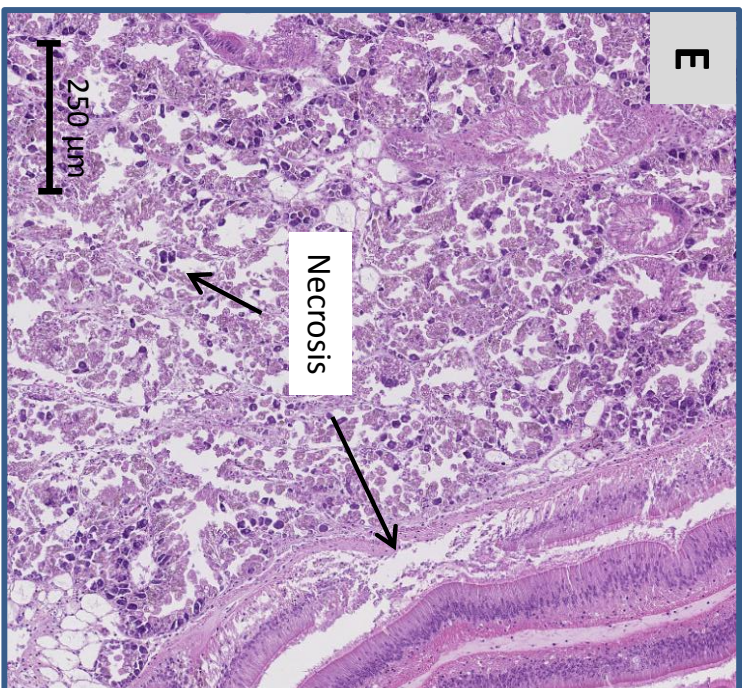
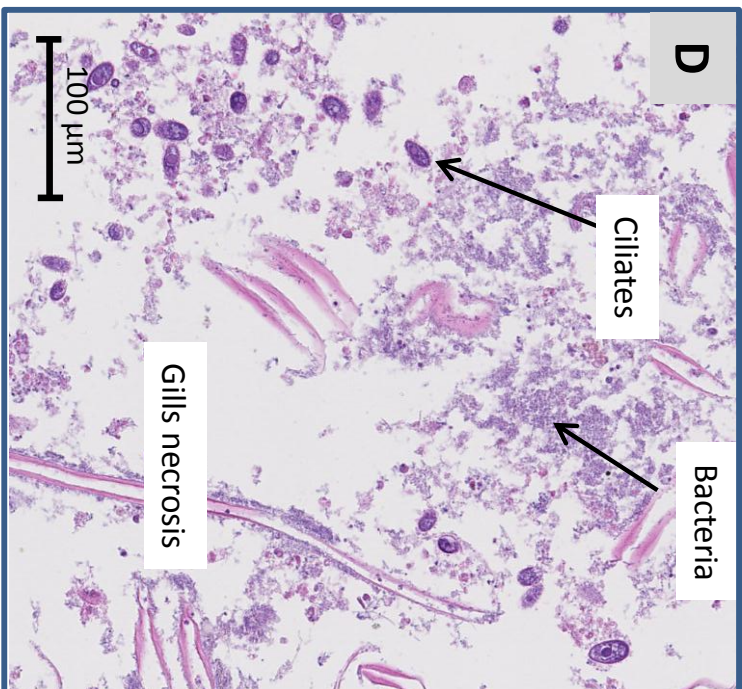
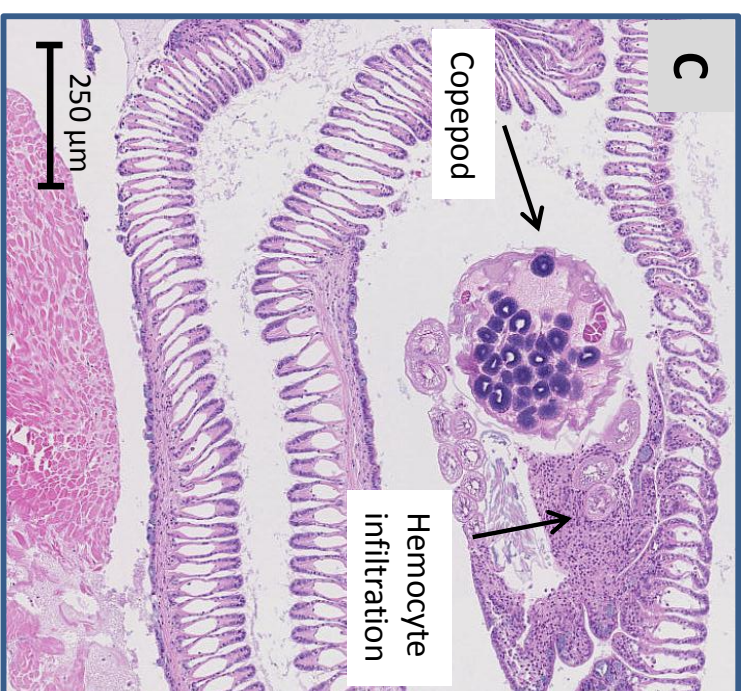
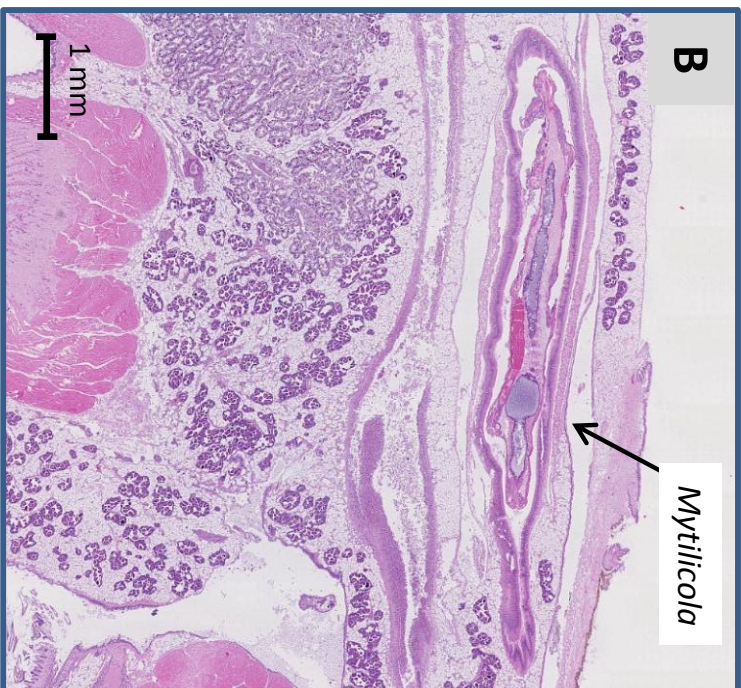
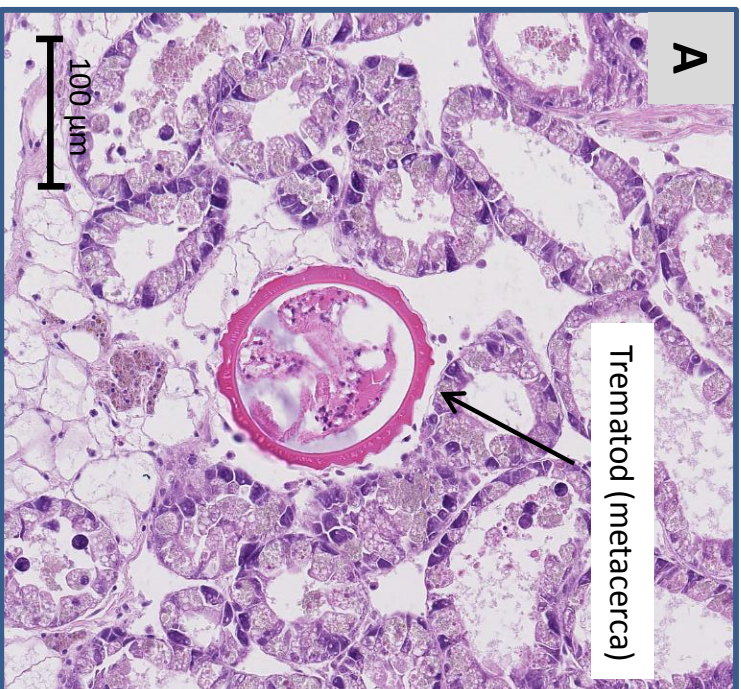


(c)











## Infection

	Absence (0)	Low (1)	Moderate (2)	High (3)
<b>Presence of noticeable protozoan</b>	40 (includ. 9M)			
<b>Trematods in digestive gland or mantle</b>	4 (includ. 2M)	21 (includ. 5 M)	15 (includ. 2M)	
<b>Copepods (<i>Mytilicola</i> sp.) in digestive light</b>	37 (includ. 9M)	3 (includ. 0M)		
<b>Ciliates in digestive gland epithelium</b>	32 (includ. 9M)	1 (includ. 0M)	7 (includ. 1M)	
<b>Ciliates in pallial cavity</b>	12 (includ. 1M)	21 (includ. 3M)	5 (includ. 4M)	2 (includ. 1M)
<b>Gregarines in mantle</b>	33 (includ. 7 M)	7 (includ. 2M)		
<b>Bacteria in gills and pallial cavity</b>	23 (includ. 0M)	8 (includ. 4M)	6 (includ. 2M)	3 (includ. 3M)
<b>Bacteria in digestive glands and esophagi</b>	30 (includ. 3M)	6 (includ. 3M)	4 (includ. 3M)	
<b>Bacteria in mantle</b>	31 (includ. 2M)	3 (includ. 1M)	6 (includ. 6M)	
<b>Necrosis of gills</b>	10 (includ. 0M)	20 (includ. 5M)	7 (includ. 1M)	3 (includ. 3M)
<b>Necrosis of digestive gland or esophagi epithelium</b>	32 (includ. 9M)	4 (includ. 1M)	4 (includ. 0M)	
<b>Hemocytic infiltrations in digestive gland, mantle or gills</b>	16 (includ. 4M)	12 (includ. 1M)	12 (includ. 4M)	
<b>Granulomas</b>	23 (includ. 9M)	10 (includ. 0M)	5 (includ. 0M)	2 (includ. 0M)