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Persistence and Recycling of Bioinsecticidal *Bacillus thuringiensis* subsp. *israelensis* Spores in Contrasting Environments: Evidence from Field Monitoring and Laboratory Experiments

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1 **Persistence and recycling of bioinsecticidal *Bti* spores in contrasting environments:**
2 **evidence from field monitoring and laboratory experiments.**

3
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28
29 Running title: Persistence of *Bti* spores in the environment

30
31

32 **Abstract**

33

34 **Sprays of commercial preparations of the bacterium *Bacillus thuringiensis* subsp.**
35 ***israelensis* (*Bti*) are widely used for the control of mosquito larvae. Despite an abundant**
36 **literature on *Bti* field efficiency on mosquito control, few studies have evaluated the fate**
37 **of spores in the environment after treatments. In the present article, two complementary**
38 **experiments were conducted to study the effect of different parameters on *Bti***
39 **persistence and recycling, in field conditions and in the laboratory. First, we monitored**
40 ***Bti* persistence in the field in two contrasting regions in France: the Rhône-Alpes region,**
41 **where mosquito breeding sites are temporary ponds under forest cover with large**
42 **amounts of decaying leaf matter on the ground, and the Mediterranean region**
43 **characterized by open breeding sites such as brackish marshes. Viable *Bti* spores can**
44 **persist for months after a treatment, and their quantity is explained both by the**
45 **vegetation type and by the number of local treatments. We found no evidence of *Bti***
46 **recycling in the field. Then, we tested the effect of water level, substrate type, salinity,**
47 **and presence of mosquito larvae on the persistence/recycling of *Bti* spores in controlled**
48 **laboratory conditions (microcosms). We found no effect of change in water level or**
49 **salinity on *Bti* persistence over time (75 days). *Bti* spores tended to persist longer in**
50 **substrates containing organic matter compared to sand-only substrates. *Bti* recycling**
51 **only occurred in presence of mosquito larvae, but was unrelated to the presence of**
52 **organic matter.**

53

54 **Keywords: *Bacillus thuringiensis* subsp *israelensis*; persistence; field; recycling; mosquito**
55 **larvae**

56

57 **Introduction**

58 The bioinsecticide *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) is often considered a safe and
59 environmentally-friendly alternative to chemical insecticides and it is increasingly used
60 worldwide for mosquito control [1]. Its toxicity for mosquito larvae is conferred by a toxic
61 crystal produced during the sporulation of the bacterium [2]. Recently, a European directive
62 on biocidal products has made *Bti* one of the few larvicides authorized for mosquito control in
63 Europe. *Bti* is usually sprayed in breeding sites as a suspension of spores and crystals that kill
64 mosquito larvae by gut disruption after ingestion [3]. It is generally characterized by low-level
65 persistence in the environment despite a huge literature reporting that its residual insecticidal
66 activity can range from a few days up to several months [4-6]. Several parameters have been
67 reported to have an impact on *Bti* toxicity such as UV light, temperature, pollution, salinity or
68 the presence of organic matter [2, 7-10]. In contrast, little is known about the parameters that
69 could influence the persistence and the possible recycling of spores in the environment.
70 Although several authors have reported a recycling of *Bti* in cadavers of mosquito larvae in
71 controlled or simulated conditions [11-13] and of *Bti*-killed mosquito larvae to scavenging
72 larvae [14], evidence for recycling under natural field conditions is scarce (but see [15, 16]).
73 One of these recycling events has been described in mosquito breeding sites from the French
74 Rhône-Alpes region in which leaf litter sampled several months after a treatment contained
75 high amounts of spores and toxins and exhibited a high toxicity for mosquito larvae [15, 17,
76 18]. Another case of *Bti* recycling has been reported in simulated conditions in water tanks,
77 which are typical breeding sites for mosquitoes in urban areas [16]. Understanding the
78 parameters that favour *Bti* persistence and recycling in various conditions of treatments and
79 environments (from large-scale spraying across breeding sites in the field to local applications
80 in water tanks in urban areas) is of high importance to ensure a sustainable and integrated use
81 of this widely used bioinsecticide.

82 In order to characterize these parameters, we first monitored persistence of spores throughout
83 the operational season in mosquito breeding sites from two contrasting French regions
84 representative of a large range of treated mosquito breeding sites and of different operational
85 practices. *Bti* has been sprayed in the French Rhône-Alpes region for several decades, but it
86 only replaced the organophosphate Temephos ten years ago in the French Mediterranean
87 region. These two regions strongly differ both in the type of mosquito breeding sites
88 (woodland freshwater temporary ponds *versus* large open areas of brackish marshes), and in
89 the operational treatments performed (Vectobac[®] WG, solid formulation applied mainly by
90 backpack spraying *versus* Vectobac[®] 12AS, liquid formulation sprayed by aircraft).

91 Then, we tested the effect of selected parameters on *Bti* persistence in laboratory controlled
92 conditions (microcosms). The influence of salinity and substrate type (sand or organic matter)
93 were tested as they are two main parameters differing between the two French regions
94 previously investigated. Moreover, considering that mosquito breeding sites often experience
95 drying/watering episodes, we also tested the effect of water level fluctuation on spore
96 persistence. As *B. thuringiensis* is an entomopathogen [19], presence of insect hosts could
97 influence its persistence and recycling. Therefore, we tested the effect of the presence of
98 mosquito larvae in two different conditions: with an organic matter substrate and in water
99 only, in order to simulate the two situations where *Bti* recycling has been reported so far [16,
100 17]. All the results are discussed regarding to *Bti* persistence in the environment and its
101 consequences on field treatments and management strategies.

102
103

104 **Materials and methods**

105 **Field sampling**

106 The field study was performed in two different areas in France, where *Bti* is applied for
107 mosquito control: Rhône-Alpes and Mediterranean region, during 3 years (2009-2011). A
108 total of 28 sites were sampled, including 16 in the Rhône-Alpes region and 12 in the
109 Mediterranean region (Fig. 1). Samplings were performed before the first *Bti* treatment of the
110 year, two days after, and then 90 and 180 days after the first treatment, corresponding to the
111 middle and end of the mosquito control season, respectively. In the Rhône-Alpes region, most
112 sites were temporary ponds under forest cover (*Alnus*, *Fraxinus*, *Quercus*, *Salix*) with large
113 amounts of decaying leaf litter accumulating on the ground, whereas Mediterranean study
114 sites were marshes with variable hydroperiod and salinity and a vegetation cover either
115 dominated by *Salicornia* (salt marshes), *Scirpus* (bulrush beds), *Juncus* (rush beds), or
116 *Phragmites* (reed beds). Characteristics of the sites are summarized in Table 1. They were
117 classified according to the vegetation cover (low: direct UV light exposure, or dense: no or
118 limited light exposure), and the *Bti* treatment history during the study season (treated or
119 untreated, and total number of treatments). For each site and each date, two superficial soil
120 samples (about 5 g each, < 1cm depth, including organic litter when present) were collected.
121 Depending on the amount of leaf litter present in the collected samples (*de visu* evaluation),
122 they were classified in three categories: null (inorganic substrate only), low (both organic and
123 inorganic substrate present in the sample), or high (leaf litter only: this was characteristic of
124 all forest sites). Samples were dried at 50°C for 48h, powdered with a planetary ball mills
125 (PM100, Retsch®, Haan, Germany), and stored at -20°C in sterile vials until use.

126

127 **Microcosm experiments**

128 Two experiments were performed in 130 L glass aquaria (microcosms) set in the laboratory at
129 room temperature (approximately 22°C), in a light–dark regimen of 16:8. The first experiment
130 was aimed at evaluating the influence of change in organic matter, water level, and salinity on
131 *Bti* persistence (Fig. 2A), while the second experiment was designed to evaluate the effect of
132 the presence of mosquito larvae on *Bti* recycling in presence of organic matter or in water
133 only (Fig. 2B).

134

135 *Experiment 1: Effect of change in water level, salinity and substrate on Bti persistence (Fig.*
136 *2A)*

137 Twenty microcosms were filled with 8 cm of autoclaved sand and clay 30% (inorganic
138 substrate) and half of them (ten microcosms) were added with 5 cm of compost and 5 cm of
139 leaf litter collected in an untreated forest breeding site in Rhône-Alpes (organic substrate,
140 conditions a-e). Sixty-five litres of tap water were added in each microcosms (hardness 15 to
141 20 terahenry (TH), pH=7) to reach 20 cm water level. Four environmental conditions were
142 examined for each substrate: salinity at 0 g.L⁻¹ (conditions a,f), salinity at 40 g.L⁻¹
143 (corresponding to the maximum salinity in a marsh pool in the Mediterranean area; conditions
144 b,g), water level maintained at 20 cm (conditions d,i), and water level fluctuating from 20 to 0
145 cm weekly (meaning a week with 20 cm followed by a week with 0 cm; conditions e,j).
146 Microcosms were treated with *Bti* at 5 L.ha⁻¹ (nominal concentration for 20 cm water depth:
147 2.5 µL.L⁻¹) and each modality was performed in 2 replicates. The 5 L.ha⁻¹ dose corresponds to
148 twice the recommended rate for aerial treatments [20]. Four untreated microcosms remained
149 as controls, two with inorganic substrate (condition h) and two with organic-enriched
150 substrate (condition c).

151

152 *Experiment 2: Effect of the presence of larvae on Bti persistence and recycling (Fig. 2B)*

153 Nine microcosms were filled with organic substrate and 65 L of tap water (hardness 15 to 20
154 TH, pH=7; conditions a-e), including one untreated microcosm as a control (condition c).
155 Nine microcosms were only filled with water (conditions f-j), including one untreated
156 microcosm as a control (condition h). In half of the microcosms (four microcosms per
157 substrate), 5000 third-instar mosquito larvae were added before *Bti* treatment (conditions
158 d,e,i,j). Such a high number of larvae was chosen in order to cover the entire substrate with
159 dead larvae after treatment. Larvae were all dead within 24 h. The laboratory “Bora Bora”
160 strain of *Aedes aegypti* was used for the experiment. Larvae were previously reared in tap
161 water and fed with standard amounts of larval food (dry dog food) in standard laboratory
162 conditions (27°C, light–dark regimen of 16:8, and 70% relative humidity) during 5 days
163 before the experiment. Two *Bti* concentrations were used for each condition: 5 L.ha⁻¹
164 (conditions a,d,f,i) and 25 L.ha⁻¹ (conditions b,e,g,j) (nominal concentration for 20 cm water
165 depth: 2.5 µL.L⁻¹ and 12.5 µL.L⁻¹, respectively). Each modality was performed in 2 replicates,
166 and two microcosms without larvae remained as controls (conditions c,h).

167

168 *Microcosms Bti treatment and sampling*

169 Microcosms were allowed to stabilize for 96 h before *Bti* application. All the microcosms
170 (except controls) were treated with a commercial *Bti* formulation (Vectobac®12AS; Valent
171 Bioscience Ins., Libertyville, IL, USA; 1200 ITU/mg, suspension concentrate), at 2.5 µL .L⁻¹
172 (corresponding to 5 L.ha⁻¹) to evaluate the effect of substrate, salinity and change in water
173 level on *Bti* persistence (experiment 1), and at two different *Bti*-concentrations (2.5 µL .L⁻¹
174 and 12.5 µL .L⁻¹ corresponding to 5 and 25 L.ha⁻¹) to evaluate the effect of mosquito larvae on
175 *Bti* persistence (experiment 2). Monitoring started just before the *Bti* treatment (day 0), and
176 was carried out until 75 days after insecticide spraying. Sampling was performed on days 0, 1,
177 2, 7, 14, 21, 28, 45, 60, and 75.

178 In microcosms with substrate, two samples consisting of 5 g of substrate were sampled at
179 each sampling date. They were dried and powdered as previously described and stored at -
180 20°C until use. In microcosms without substrate, water was stirred, and twice 250 mL of
181 water were taken, without subsequent refilling of the microcosm. Water samples were filtered
182 through a fiberglass filters (0.2 µm pores, Isopore™ membrane filters, Millipore, Billerica,
183 MA, USA) and filters were stored at -20°C in sterile vials.

184 On each sampling date, the water temperature, dissolved oxygen, salinity and pH were
185 measured in every microcosm at ca. 5 cm below the water surface, using portable apparatuses
186 (Wissenschaftlich-Technische-Werkstätten –WTW, Champagne au Mont d’Or, France) to
187 control the stability of the systems. Collected data are not presented as they remained stable
188 during the course of the experiment.

189

190 *Colony counting*

191 One gram from each dried sample (or filter) was suspended into 10 mL of TSB (Trypticase
192 Soy Broth) for 60 min. The suspension was then heated to 80°C for 30 min, such that only
193 bacterial spores survived [15, 21]. Tenfold dilutions were made in TSB (10⁻¹ and 10⁻²), and
194 200 µL of each dilution were plated on nutrient agar in duplicate. Plates were incubated at 30
195 ± 2°C for 24 h under aerobic conditions. *Bacillus* colonies were identified and counted by
196 their morphological appearance. Colonies that were beige, with irregular edges and an “ice
197 crystal” appearance were considered as *Bacillus thuringiensis* [22]. To verify these colonies
198 were indeed *Bacillus thuringiensis* subsp. *israelensis* we randomly picked 22 colonies and
199 bacilli were incubated with rabbit antiserum specific for H14 serotype (kindly provided by
200 Christina Nielsen-Leroux) during 1 hour at room temperature. After 3 washes with PBS and
201 1h incubation with FITC-conjugated goat anti-rabbit Ig (Southern biotech, USA), stained
202 bacteria were observed using fluorescent microscopy (BX41 Olympus, France) as described

203 in [15]. All the colonies identified morphologically as being *Bti* were detected as *Bti* by
204 immunology.

205

206 ***Data analysis***

207 Data normality was tested using Shapiro–Wilk test, and the homogeneity of variances
208 between treatments was tested using Bartlett’s test. Because *Bti* colony forming units (CFU)
209 counts were not normally distributed, but followed a quasi-poisson distribution, we first
210 performed non-parametric tests (Mann-Whitney and Kruskal Wallis tests) on each separate
211 explanatory factor. Because non-parametric tests do not allow testing simultaneously different
212 factors and their interactions, we then performed generalized linear models adapted to our
213 non-normally distributed, overdispersed, count datasets (quasi-poisson family, log link). In
214 experiments 1 and 2, the microcosm effect was considered as a random factor. Control
215 microcosms (without *Bti*) that contained no colonies were not included in further statistical
216 analysis. All tests were performed using R software version 2.15.1 [24]. Significance was
217 accepted at $\alpha = 0.05$ for all tests.

218

219

220 **Results**

221 *Field study*

222 Most of the collected field samples did not contain any viable *Bti* spores, and some samples
223 from untreated sites contained viable *Bti* spores. However, treated sites contained an order of
224 magnitude more *Bti* viable spores than untreated sites (Fig. 3A), and the treatment effect
225 explained 7.5% of the total deviance (Table 2). Furthermore, CFU counts and the total
226 number of treatments in a given site were positively correlated (Spearman's correlation
227 coefficient $r_s = 0.22$, $p = 3 \cdot 10^{-7}$). Vegetation type explained 8.8% of the total deviance, with
228 more viable spores found in meadows, rush beds and forests, than in reed beds, salt marshes
229 and bulrush beds (Fig. 3B). In order to disentangle the respective roles of insecticide pressure
230 (total number of treatments), vegetation cover, and organic matter present on the ground to
231 explain *Bti* spore persistence, a generalized linear model including the three factors and all
232 possible interactions as explanatory variables was fitted to the number of colonies counted
233 (Table 3). The factor 'Vegetation Cover' was significant and explained 1.7% of total
234 deviance, with more *Bti* spores found under a dense vegetation cover. Although the factor
235 'Insecticide pressure' alone was not significant, the interactions between 'Insecticide
236 pressure' and 'Leaf litter' factors, and between 'Insecticide pressure', 'Leaf litter' and
237 'Vegetation Cover' were significant and explained respectively 17% and 2.5% of total
238 deviance. More viable *Bti* spores were found with increasing number of treatments in sites
239 with high leaf litter content, corresponding to forest sites characteristics of the Rhône-Alpes
240 region; the correlation between the number of *Bti* treatments and CFU was positive in both
241 regions, but only significant in the Rhône-Alpes region (Spearman's correlation coefficient r_s
242 $= 0.42$, $p = 7.25 \cdot 10^{-13}$ in the Rhône-Alpes region and $r_s = 0.11$, $p = 0.09$ in the Mediterranean
243 region).

244

245 *Microcosm study*

246 From day 0 to day 7, *Bti* viable spores were significantly more abundant in the microcosms
247 containing an organic substrate (compost and leaf litter) than in microcosms with inorganic
248 substrate only (mixture of sand and clay only) (Fig. 4A; date by date analysis, Mann-Whitney
249 tests, all $p < 0.001$). However, this effect was no longer significant after day 7 due to the high
250 variability between replicates (date by date analysis, Mann-Whitney tests, all $p > 0.1$). There
251 was no significant effect of water salinity (Fig. 4B) or water level fluctuation (Fig. 4C) on *Bti*
252 persistence (date by date analysis, all Mann-Whitney tests $p > 0.05$). In order to determine
253 which factors and combination of factors best explain the *Bti* spore persistence patterns
254 observed over time, we performed a generalized linear mixed model (GLMM) including
255 sampling dates, substrate type (inorganic or organic), water salinity (40 g.L⁻¹ or 0 g.L⁻¹), and
256 water level (maintained or fluctuating), as fixed factors, and microcosm as random effect to
257 explain the number of *Bti* colonies counted (Table 3). Only the factors 'Date' and 'Substrate'
258 had a significant effect ($p = 2.347 \cdot 10^{-07}$ and $p = 0.0355$, respectively), which is due to a
259 significant increase in CFU.g⁻¹ observed just after treatment (day 1) followed by a non-
260 significant decrease in CFU.g⁻¹ from day 2 to the end of the experiment (day 75). The second-
261 and third-order interactions were not significant (Table 4).

262

263 Change in *Bti* colonies abundance over time with or without mosquito larvae, at two different
264 *Bti*-concentrations, was monitored (Fig. 5). The number of *Bti* colonies was significantly
265 higher in presence of mosquito larvae from day 21 to the end of the study period, except at
266 day 60 (Kruskal-Wallis test, $p > 0.05$), irrespective of the *Bti* concentration applied. This is in
267 contrast to the microcosms without mosquito larvae where *Bti* colonies remained low for both
268 *Bti* concentrations.

269 GLMM including sampling dates, substrate type (organic substrate with leaf litter, or water
270 only), presence or absence of mosquito larvae, and concentration (concentration of *Bti*: 2.5 or
271 12.5 $\mu\text{L} \cdot \text{L}^{-1}$) as fixed factors and microcosm as random factor was fitted to the number of
272 colonies counted (Table 5). The factors 'Date' and 'Larvae' were highly significant, with a
273 significant increase in CFU just after treatment (day 1) followed by a maximal increase by
274 two orders of magnitude in CFU after 28-45 days in presence of larvae. Although there was
275 no effect of 'Substrate' or 'Treatment' factors alone, there were significant interactions
276 between 'Date' and 'Substrate' (with maximum CFU counts at day 28 in organic matter and
277 at day 45 in water), between 'Date' and 'Treatment' and between 'Substrate' and
278 'Concentration' factors. All other interactions between factors were not significant (Table 5).
279
280
281

282 **Discussion**

283 ***Fate of Bti spores in the field***

284 The number of viable *Bti* spores recovered from field samples was highly variable, ranging
285 from 0 up to 10^5 , with an average of 10^4 CFU.g⁻¹. This is comparable to previous studies, with
286 10^4 - 10^6 CFU.g⁻¹ in a temporary flooded salt marsh in the Mediterranean region treated twice
287 with *Bti* during the season [13], and 10^3 - 10^6 CFU.g⁻¹ in a natural wetland treated for 22 years
288 in Switzerland [8, 12]. In a given site, the number of CFU.g⁻¹ found was correlated to the total
289 number of treatments performed the year of the study. The same pattern was observed in a
290 Swiss natural wetland where *Bti* abundance was explained by the number of treatments of the
291 year, with no evidence for a cumulative effect from one year to the next [12]. These results
292 suggest that *Bti* spores can persist for months during the treatment season (from spring to
293 autumn), but the amount of viable overwintering spores is too low to significantly affect the
294 amount of spores the following year. In some of our study sites, viable *Bti* spores were found
295 before the first treatment occurred, as well as in some untreated sites, but in much lower
296 amount than in treated sites. These spores might be either overwintering spores from
297 treatments performed the year before, or indigenous *Bti* strains (unrelated to *Bti* treatment).
298 Whatever the origin of these *Bti* spores, *Bti* treatment significantly increased the number of
299 spores locally recovered.

300 Our results revealed that vegetation cover was a factor affecting *Bti* spores persistence, with
301 more colonies found in dense compared to low cover sites (2344 versus 1196 CFU .g⁻¹ in
302 dense and low cover sites, respectively). Considering that UV light is known to decrease *Bti*
303 efficacy [18], this result suggests that the presence of dense vegetation cover may protect the
304 integrity of *Bti* spores by filtering UV light. This protective effect seems to compensate a
305 possible reduced amount of *Bti* reaching the ground under dense vegetation cover. More than
306 vegetation cover, the main factor explaining *Bti* spore persistence in the field was the
307 interaction between insecticide pressure and leaf litter content on the ground, explaining up to
308 17% of total deviance. More precisely, viable *Bti* spore number was positively correlated to
309 the number of treatments only in the Rhône-Alpes region, where all sites are characterized by
310 dense vegetation cover and high litter content. By contrast, in sites of the Mediterranean
311 region, with no or low amounts of organic matter on the soil, insecticide pressure and CFU
312 counts were not correlated, suggesting that in the absence of organic matter on the ground,
313 there is no accumulation of spores throughout the treatment season. The leaf litter present on
314 the ground may favor *Bti* spore accumulation on the substrate surface by protecting them from
315 being flooded away, and might also have a role in UV protection. Furthermore, the interaction
316 effect is to be related not only to difference in the vegetation cover and leaf litter content on
317 the ground between the two regions, but also to difference in the intensity of insecticide
318 pressure; the maximum number of treatments in one single site is 25 in Mediterranean region,
319 compared to 6 in Rhône-Alpes region. Such difference can be explained both by the treatment
320 strategy (i.e. treatments for mosquito control are applied only when mosquito larvae are
321 observed) and the nature of the treated wetlands. Indeed, most mosquito breeding sites from
322 the Mediterranean region are highly temporary flooded areas with a succession of
323 drying/watering events over the season, requiring many treatments per year for one site.
324 Breeding sites from the Rhône-Alpes region generally experience less hydrological variations,
325 resulting in a lower insecticide pressure than in the Mediterranean region. Moreover, the
326 Mediterranean mosquito control agency sprays *Bti* in large open areas by plane while most
327 breeding sites from the Rhône-Alpes region are small woodland temporary ponds sprayed by
328 walking agents.

329

330 ***Effect of salinity and water level fluctuation on Bti spores persistence***

331 Salinity was recently shown to have a small but significant negative impact on the toxicity of
332 *Bti* to *Ae. aegypti* and *Anopheles gambiae* larvae [23, 24] but the nature of the interaction
333 between salinity and *Bti* is still unknown. No study designed to evaluate the impact of salinity
334 on *Bti* spore persistence in controlled conditions has been published thus far. Our results show
335 that *Bti* spore persistence is not influenced by salinity. Therefore, salinity may act directly on
336 mosquito larvae, maybe by affecting their feeding rate, or on the crystal, potentially affecting
337 toxin stability or tridimensional toxins conformation, but it has no direct effect on the viability
338 of *Bti* spores.

339 Many mosquito breeding sites are temporary pools subjected to frequent episodes of
340 drying/watering. A previous study showed that the fluctuation of the water level had no
341 significant impact on *Bti* toxins persistence in leaf litter [17] and the present study shows that
342 it also has no effect on spore persistence. Altogether, these results suggest that the water level
343 fluctuation often experienced by mosquito breeding sites does not affect directly the efficacy
344 and persistence of *Bti*.

345

346 ***Parameters influencing Bti recycling***

347 Although *Bti* spores can persist for months in soil [22, 25], *Bti* is an insect pathogen and its
348 vegetative cells cannot proliferate in soils without a suitable insect host [19, 26]. Recycling of
349 *Bti* in larval cadavers (*i.e.* germination, proliferation and sporulation) in the laboratory and in
350 simulated habitats have been reported by several authors [11-13] but despite an intensive and
351 wide scale use of commercial formulations that include both viable spores and toxic crystals,
352 evidence for *Bti* recycling under natural field conditions is scarce. To our knowledge, only
353 two reports of *Bti* recycling in field conditions have been documented; the first one occurred
354 in a site rich in organic matter in the Rhône-Alpes region where a significant increase of
355 larval toxicity [18], spore number [15] and toxin quantity [17] was observed several months
356 after *Bti* treatment. However, despite the 3-years large-scale sampling performed in the
357 present study, we were not able to detect any new case of recycling in the field, neither in the
358 Rhône-Alpes region, nor in the Mediterranean region. The design of our field study was not
359 optimal to detect *Bti* recycling as we monitored operational sites under continuous insecticide
360 pressure throughout the season, with the last sampling occurring one month after the last
361 treatment. To demonstrate that recycling occurred in the field would require monitoring sites
362 for a longer time after the last treatment. This was done in our experimental design where one
363 single *Bti* treatment was performed, followed by regular sampling and spore counting up to 75
364 days after treatment. The second case of recycling reported in the literature [16] was observed
365 in water containers, which represents a typical mosquito breeding sites in *Bti*-treated urban
366 areas. In this study, recycling was evidenced by observing a 100-fold increase of bacterial
367 concentration (to reach 10^6 spores per mL) from day 30 to day 180 after treatment [16].

368 By reproducing these two conditions where *Bti* recycling has been observed (with substrate
369 rich in organic matter and in water only), we showed that *Bti* recycling occurred only in
370 presence of mosquito larvae, both in organic rich and water microcosms. Evidence for
371 recycling is given by the increase by 2 orders of magnitude in the CFU counts 28-45 days
372 after treatment, with an increase of up to $2.5 \cdot 10^5$ CFU $\cdot g^{-1}$ as compared to an average of 10^3
373 CFU $\cdot g^{-1}$ just after treatment and throughout the experiment (75 days) in microcosms without
374 larvae. This 30-days delay between *Bti* treatment and recycling seems to be the time required
375 for the full *Bti* life cycle to complete; that is, the time required for the toxins to be ingested by
376 larvae, activated in the insect alkaline midgut, to bind to specific receptors and disrupt the
377 midgut epithelium, to penetrate into the insect tissues, germinate, and proliferate as vegetative
378 cells [3]; sporulation is a secondary process (when nutrients from larval tissues are no more
379 available) which involves several stages including formation of a composite proteic crystal

380 and of a forespore, formation of exosporium, cortex and spore coats, and spore maturation
381 [27].

382 The same pattern of recycling was found with the two *Bti* concentrations tested, indicating
383 that the first concentration, which corresponds to twice the recommended operational dose,
384 was sufficient to kill all the larvae present in the aquarium, leading to a saturation of the
385 resources available. Therefore, increasing the quantity of *Bti* sprayed did not result in an
386 increase of the overall bacterial division success. However, the significant interaction
387 observed between dates and *Bti* concentration reflects that *Bti* was more rapidly ingested by
388 larvae when it was provided in excess. A new experiment allowing measuring the uptake and
389 quantity of *Bti* in larvae over time must be designed to validate this hypothesis, which could
390 not be tested in the present study due to the fast decomposition of larval cadavers (in 24 to 48
391 hours).

392

393 ***The effect of the organic matter***

394 The organic matter is known to be a major factor influencing both the efficacy of *Bti* and the
395 persistence of its mosquitocidal activity. Indeed, *Bti* toxicity was shown to decrease with
396 water turbidity and in organic rich habitats because toxins are rapidly denatured and/or bound
397 to organic matter [2]. Furthermore, it has recently been shown that *Bti* toxins behave
398 differently in presence of leaf litter, which may partly explain the rapid loss of toxicity
399 observed in presence of leaf litter [10, 17]. In contrast to toxin persistence, the present study
400 shows that organic matter (including leaf litter) is a factor favouring *Bti* spore persistence.
401 Indeed, both microcosm and field experiments revealed a positive effect of organic matter on
402 spore persistence. The presence of leaf litter on the substrate may immobilize *Bti* spores and
403 prevent them from being flowed away, favouring their accumulation throughout repeated
404 treatments. Leaf litter might also contribute to protect *Bti* spores from direct UV light.

405 No effect of the substrate was observed on *Bti* recycling in larvae as similar recycling patterns
406 were observed in microcosms with or without organic matter. The significant interaction
407 between substrate and dates is only due to the fact that more spores were found just after
408 treatment in the microcosms with organic matter, confirming the positive effect of organic
409 matter on spore persistence.

410 Altogether, these results show that the organic matter has a contrasting effect on *Bti*
411 persistence, decreasing its efficacy and the persistence/bioavailability of its toxins [10] but
412 increasing the persistence of the spores over time (present study). This is consistent with
413 previous studies conducted on *Btk* (*Bacillus thuringiensis* subsp *kurstaki*) where no
414 correlation was found between spores and toxins recovered in soils after spraying [28].
415 Crystals and spores are two highly different structures; the first being a conglomerate of
416 proteins responsible for toxicity, while the latter is a survival form of the bacteria. Therefore,
417 it is somewhat unsurprising that their interaction with the complex structure of the organic
418 matter differ. Finally, although organic matter by itself does not directly promote *Bti* recycling
419 in absence of mosquito larvae, the presence of organic matter increased spores persistence,
420 and therefore the probability for *Bti* spores to be ingested by mosquito larvae and to
421 proliferate.

422

423 **Conclusion**

424 Our results reveal that viable *Bti* spores can persist for months in the environment after a
425 treatment, and that their quantity is explained both by the number of local treatments and by
426 the type of vegetation. Salinity and water level fluctuation did not influence *Bti* spores
427 persistence in controlled conditions, while the presence of organic matter was a major
428 parameter affecting spores persistence both in the field and in the laboratory. Finally, no
429 evidence for *Bti* recycling was found in the field; but we experimentally showed that the

430 presence of larvae is necessary for *Bti* recycling, irrespective of the presence of organic
431 matter.

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441
442

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533 **Table 1. Main characteristics of the sites studied. The region, name of site, year of**
534 **sampling, treatment modality, the main vegetation type and vegetation cover (dense or**
535 **low) are indicated. Insecticide pressure (number of treatments) and amount of leaf litter**
536 **in soil samples are also indicated. The code indicated in the third column corresponds to**
537 **the code in Fig. 1.**

Region	Study Sites	Code	Sampling year	Treatment	Vegetation	Cover	Insecticide pressure	Leaf litter
Mediterranée	Beauchamp	BC	2009	Treated	Bulrush bed	low	3	low
	Cabane du Roc	CR	2009	Treated	Salt marsh	low	8	null
	Caisse de Mort	CM	2009	Treated	Salt marsh	low	12	null
	Les Enfores 1	EN	2009	Treated	Salt marsh	low	3	null
	Manade Blattière	MBI	2009	Treated	Meadow	low	5	low
	Mas Badet	MB	2009	Untreated	Salt marsh	low	0	null
	Mas d'Icard	MI	2009	Untreated	Reed Bed	dense	0	low
	Tour Carbonnière	TC	2009	Treated	Salt marsh	low	2	null
	Triangle aux Anes	TA	2009	Treated	Rush bed	dense	2	null
	La Carbonelle	CA	2010	Treated	Meadow	low	3	null
	La Douane	DO	2010	Treated	Rush bed	dense	25	null
	Les Enfores 2	EN	2010	Treated	Reed bed	dense	6	low
	Embouchac	EM	2011	Treated	Salt marsh	low	10	null
	Les Enfores 2	EN	2011	Treated	Reed bed	dense	2	low
Rhône-Alpes	Albens 1	AL	2010	Untreated	Forest	dense	0	high
	Albens 2	AL	2010	Untreated	Forest	dense	0	high
	Albens 3	AL	2010	Treated	Forest	dense	6	high
	Albens 4	AL	2010	Treated	Forest	dense	6	high
	Château-Gaillard	CG	2010	Treated	Forest	dense	5	high
	Etremblières	ET	2010	Untreated	Reed bed	dense	0	low
	Gaillard 1	GA	2010	Treated	Forest	dense	6	high
	Gaillard 2	GA	2010	Treated	Forest	dense	5	high
	La Verpillière	VE	2010	Treated	Forest	dense	6	high
	Reignier	RE	2010	Untreated	Forest	dense	0	high
	Saint-Maurice-de-Gourdans	SMG	2010	Treated	Forest	dense	5	high
	Saint-Maurice-de-Remens	SMR	2010	Untreated	Forest	dense	0	high
	Scentrier 1	SC	2010	Treated	Forest	dense	5	high
	Scentrier 2	SC	2010	Treated	Forest	dense	4	high
	Solaize	SO	2010	Treated	Forest	dense	2	high
Ternay	TE	2010	Untreated	Forest	dense	0	high	

539 **Table 2. Analysis of the deviance table of GLM (quasi-poisson family, link=log) fitting**
 540 **CFU counts per gram in the field samples to two factors added sequentially (first to last):**
 541 **Treatment modality (treated or untreated) and Vegetation type (salt marsh, meadow,**
 542 **bulrush bed, rush bed, reed bed and forest). Chi-square *p* values indicate the level of**
 543 **significance of the various factors in the model as compared to the Null model (no effect**
 544 **included); *p* values < 0.05 are in bold characters**
 545

Factor	Df	Deviance	Chi-square <i>p</i> value
Null	519	4452895	
Treatment	1	338419	4.529 10⁻⁵
Vegetation type	5	393681	1.652 10⁻³
Treatment × Vegetation type	2	8238	0.816

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550 **Table 3. Analysis of the deviance table of GLM (quasi-poisson family, link=log) fitting**
 551 **CFU counts per gram in the field samples to three factors added sequentially (first to**
 552 **last): Insecticide pressure (expressed as the number of treatment/year/study site),**
 553 **Vegetation cover (low or dense); Leaf litter (null, low, and high). Chi-square *p* values**
 554 **indicate the level of significance of the various factors in the model as compared to the**
 555 **Null model (no effect included); *p* values < 0.05 are in bold characters**
 556

Factor	Df	Deviance	Chi-square <i>p</i> value
Null	519	4452895	
Insecticide pressure	1	245	0.899
Vegetation cover	1	78212	0.024
Leaf litter	2	60449	0.141
Vegetation cover × Leaf litter	1	56730	0.054
Insecticide pressure x Leaf litter	2	759338	1.98 10⁻¹¹
Insecticide pressure × Vegetation cover	1	16969	0.293
Insecticide pressure x Leaf litter x Vegetation cover	1	112823	6.8 10⁻³

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560 **Table 4. Analysis of the deviance table of GLMM fitting *Bti* CFU counts to four**
 561 **explanatory factors: Date: sampling dates; Substrate: inorganic substrate, and substrate**
 562 **with leaf litter; Salinity: water salinity (40 g.L⁻¹ or 0 g.L⁻¹); Water level: maintained or**
 563 **fluctuating. Microcosm effect was included in the model as a random effect (experiment**
 564 **1 of microcosms). Chi-square *p* values indicate the level of significance of the various**
 565 **factors in the model; *p* values < 0.05 are in bold characters.**
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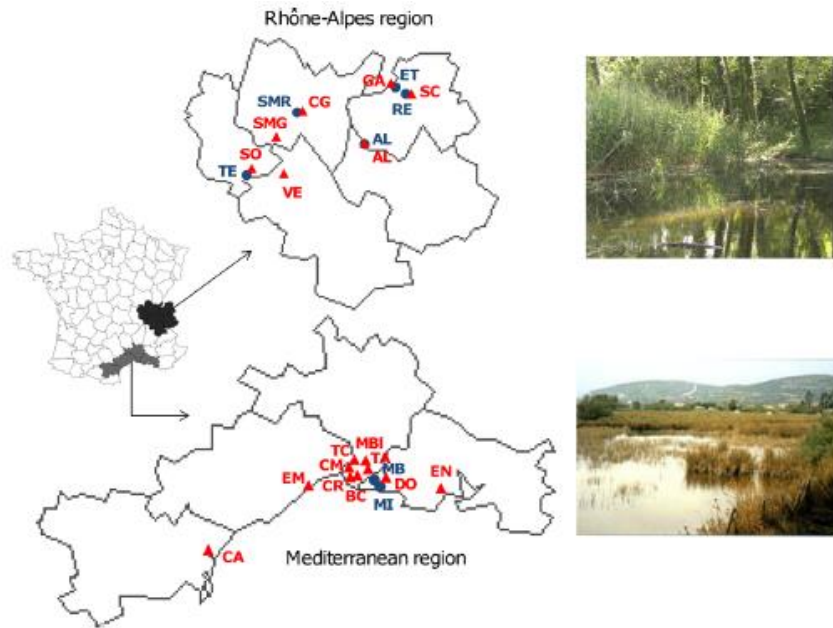
Factor	Chisq	Df	Chi-square <i>p</i> value
Date	48.2012	9	2.342e-07
Substrate	4.4208	1	0.0355
Salinity	1.0788	1	0.2990
Water height	0.0993	1	0.7527
Date × Substrate	3.3583	9	0.9484
Date × Salinity	4.3859	9	0.8842
Date × Water height	15.3338	9	0.0822
Substrate × Salinity	0.0304	1	0.8616
Substrate × Water height	0.0063	1	0.9366
Date × Substrate × Salinity	0.3544	9	0.9999
Date × Substrate × Water height	1.4954	9	0.9972

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569 **Table 5. Analysis of the deviance table of GLMM model fitting *Bti* CFU counts to four**
570 **fixed explanatory factors (experiment 2 of microcosms). Date: sampling dates;**
571 **Substrate: substrate with leaf litter and water only; Larvae: with or without mosquito**
572 **larvae; Concentration: *Bti* at 2.5 or 12.5 $\mu\text{L.L}^{-1}$. Microcosm effect was included in the**
573 **model as a random effect. Chi-square *p* values indicate the level of significance of the**
574 **various factors in the model; *p* values < 0.05 are in bold characters**
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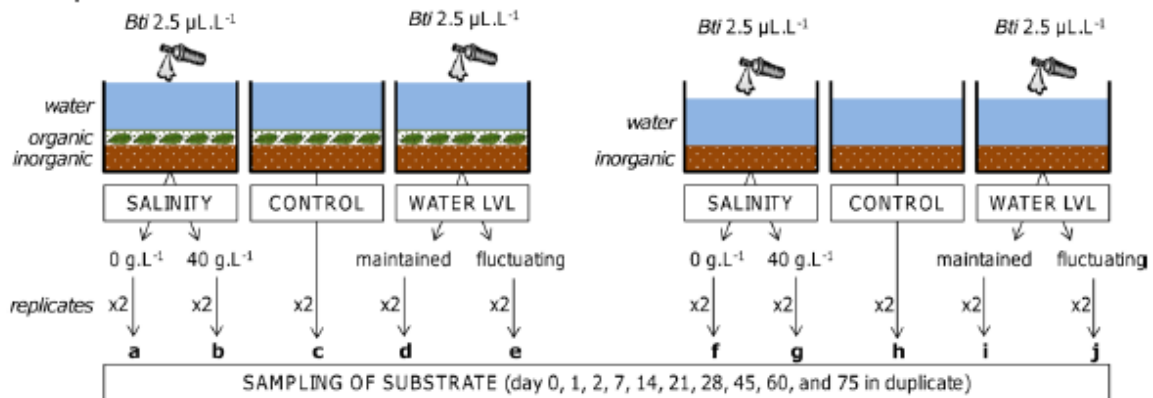
Factor	Chisq	Df	Chi-square <i>p</i> value
Date	98.1028	9	< 2.2e-16
Substrate	0.2787	1	0.5975
Larvae	15.6950	1	7.442 10⁻⁵
Concentration	0.8333	1	0.3613
Date × Substrate	48.1673	9	2.377 10⁻⁷
Date × Larvae	12.4196	9	0.1907
Date × Concentration	36.5869	9	3.118 10⁻⁵
Substrate × Larvae	2.6577	1	0.1030
Substrate × Concentration	7.0542	1	0.0079
Larvae × Concentration	0.0372	1	0.8471
Date × Substrate × Larvae	2.9440	9	0.9665
Date × Substrate × Concentration	6.8954	9	0.6480
Date × Larvae × Concentration	3.0020	9	0.9642
Substrate × Larvae × Concentration	0.3705	1	0.5427
Date × Substrate × Larvae × Concentration	0.5458	9	0.9999

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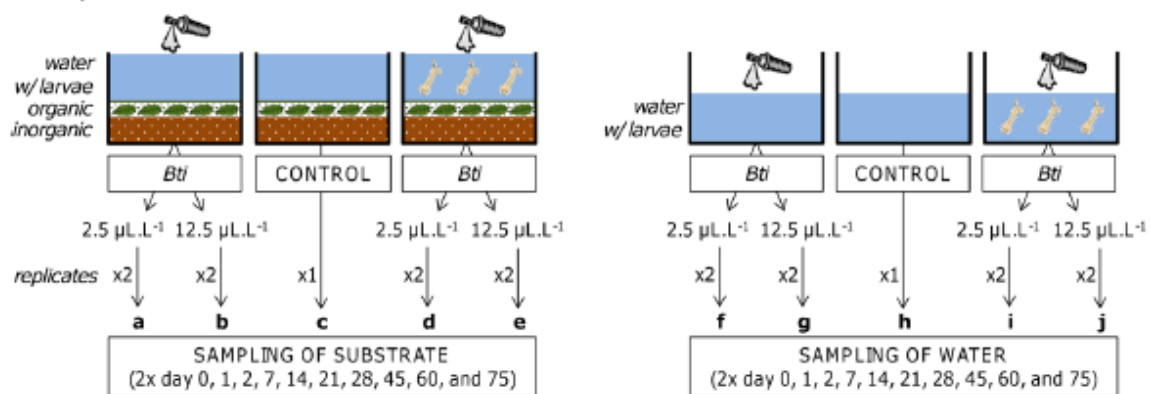


578
 579 **Fig. 1.** Localization of the mosquito breeding sites sampled, including 16 in the French
 580 Rhône-Alpes region (upper part of the figure) and 12 in the Mediterranean region (lower part
 581 of the figure). Some sites were geographically so close that they cannot be distinguished on
 582 the map (eg, AL, EN) but they differed for the number of treatments and/or vegetation type.
 583 The treated sites are indicated by a red triangle and the untreated sites by a blue circle. The
 584 correspondence of the sites names is indicated in the Table 1. A picture of a representative
 585 breeding site (*i.e.* woodland ponds enriched in organic matter *versus* open areas of salt water
 586 on a sandy substrate for the Rhône-Alpes and Mediterranean region, respectively) is shown at
 587 the right part for each region.
 588

a experiment 1

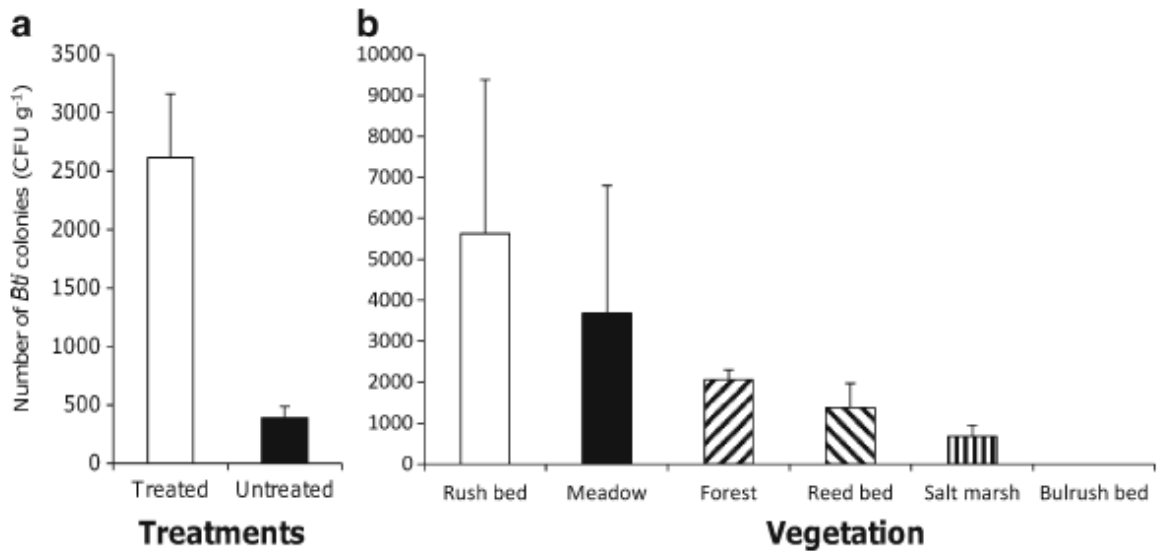


b experiment 2

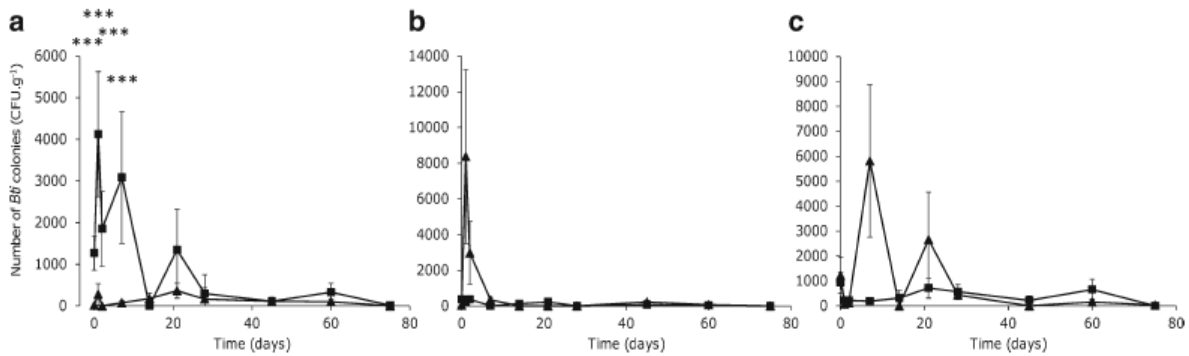


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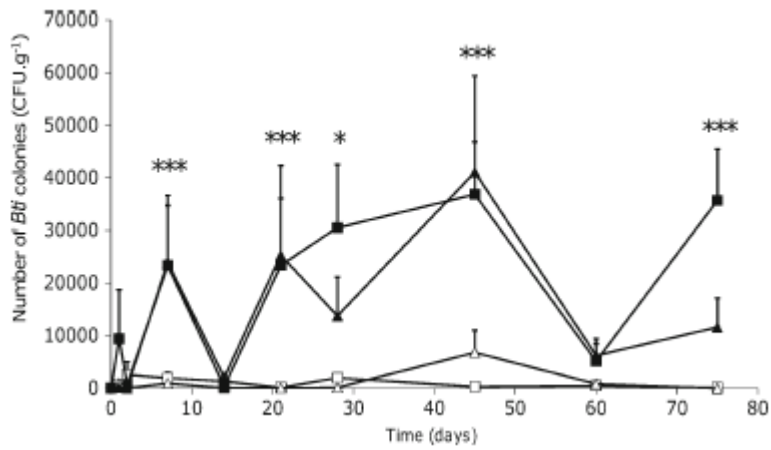
591 **Fig. 2.** Illustration of the experimental design of microcosms for evaluating the influence of
592 (A) the presence of organic matter, the salinity (conditions a,b,f,g), the water level fluctuation
593 (conditions d,e,i,j) and (B) the presence of larvae, with organic substrate (conditions a-e) or in
594 water only (conditions f-j), on the fate of *Bti*. Commercial *Bti* was sprayed at a concentration
595 of 2.5 µL.L⁻¹ (experiment 1 (A) and experiment 2 (B), conditions a,d,f,i) or 12.5 µL.L⁻¹
596 (experiment 2 (B), conditions b,e,g,j). Controls consisted in untreated aquaria (conditions c
597 and h). All conditions were performed in duplicate, except for controls in experiment 2. Ten
598 samplings were performed in duplicate for each condition and each experiment.
599



600
 601 **Fig. 3.** Quantity of *Bti* spores (mean \pm SE), expressed as colony forming unit (CFU) per gram
 602 of soil, in function of **A.** *Bti* treatment (Mann-Whitney test, $p < 0.001$) and **B.** Main vegetation
 603 type (Kruskall-Wallis test, $p < 0.001$)
 604



605
 606 **Fig. 4.** Quantity of *Bti* spores (mean \pm SE), expressed as colony forming unit per gram of soil,
 607 in function of three parameters (experiment 1 of microcosms). **A.** Influence of the substrate
 608 type on *Bti* abundance (inorganic, triangles; organic, squares). **B.** Influence of the salinity on
 609 *Bti* abundance (0 g.L⁻¹ of salt, triangles; 40 g.L⁻¹, squares). **C.** Influence of the water level on
 610 *Bti* abundance (fixed, triangles; fluctuating, squares). Mann-Whitney tests for each sampling
 611 date and for each modality: ***: $p < 0.001$.
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Fig. 5. Quantity of *Bti* spores (mean \pm SE), expressed as colony forming unit per gram of substrate, in the microcosms treated with *Bti* at 2.5 (triangle) and 12.5 $\mu\text{L.L}^{-1}$ (square), with (dark) or without (white) *Ae. aegypti* larvae (experiment 2 of microcosms). Kruskal-Wallis test: *: $p < 0.05$; ***: $p < 0.001$.