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

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Running title: Persistence of *Bti* spores in the environment

Abstract

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

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

Introduction

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

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

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

Materials and methods

Field sampling

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

Microcosm experiments

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

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

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

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

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

Microcosms Bti *treatment and sampling*

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

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

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

Colony counting

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

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

Data analysis

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

Results

Field study

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

Microcosm study

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

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

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

Discussion

Fate of Bti spores in the field

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

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

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

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

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

Parameters influencing* Bti *recycling

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

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

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

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

The effect of the organic matter

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

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

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

Conclusion

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

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

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

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

Factor	Df	Deviance	Chi-square *p* 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

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

Factor	Df	Deviance	Chi-square *p* 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	**$6.8\ 10^{-3}$**

Table 4. Analysis of the deviance table of GLMM fitting *Bti* CFU counts to four explanatory factors: Date: sampling dates; Substrate: inorganic substrate, and substrate with leaf litter; Salinity: water salinity (40 g.L^{-1} or 0 g.L^{-1}); Water level: maintained or fluctuating. Microcosm effect was included in the model as a random effect (experiment 1 of microcosms). Chi-square *p* values indicate the level of significance of the various factors in the model; *p* values < 0.05 are in bold characters.

Factor	Chisq	Df	Chi-square *p* 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

Table 5. Analysis of the deviance table of GLMM model fitting *Bti* CFU counts to four fixed explanatory factors (experiment 2 of microcosms). Date: sampling dates; Substrate: substrate with leaf litter and water only; Larvae: with or without mosquito larvae; Concentration: *Bti* at 2.5 or 12.5 µL.L^{-1}. Microcosm effect was included in the model as a random effect. Chi-square *p* values indicate the level of significance of the various factors in the model; *p* values < 0.05 are in bold characters

Factor	Chisq	Df	Chi-square *p* 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

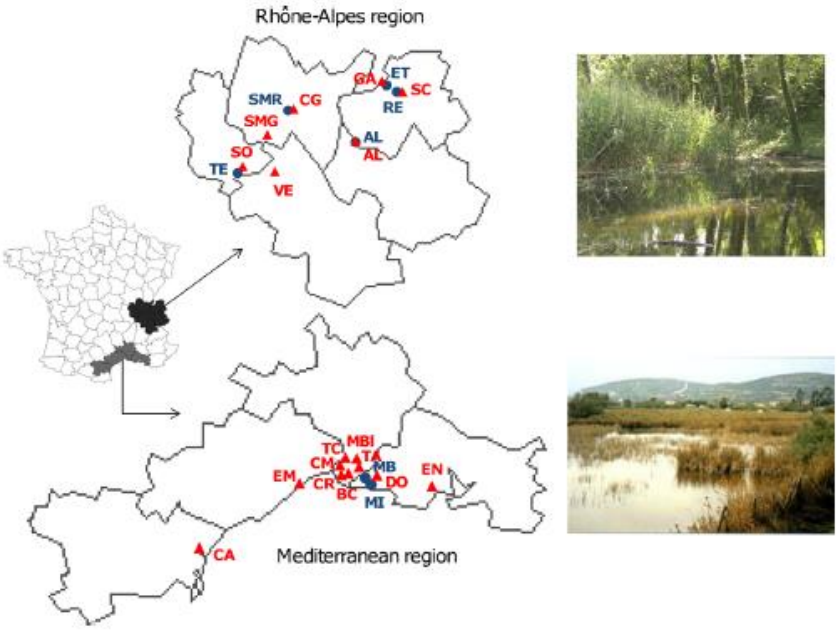

Fig. 1. Localization of the mosquito breeding sites sampled, including 16 in the French Rhône-Alpes region (upper part of the figure) and 12 in the Mediterranean region (lower part of the figure). Some sites were geographically so close that they cannot be distinguished on the map (eg, AL, EN) but they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

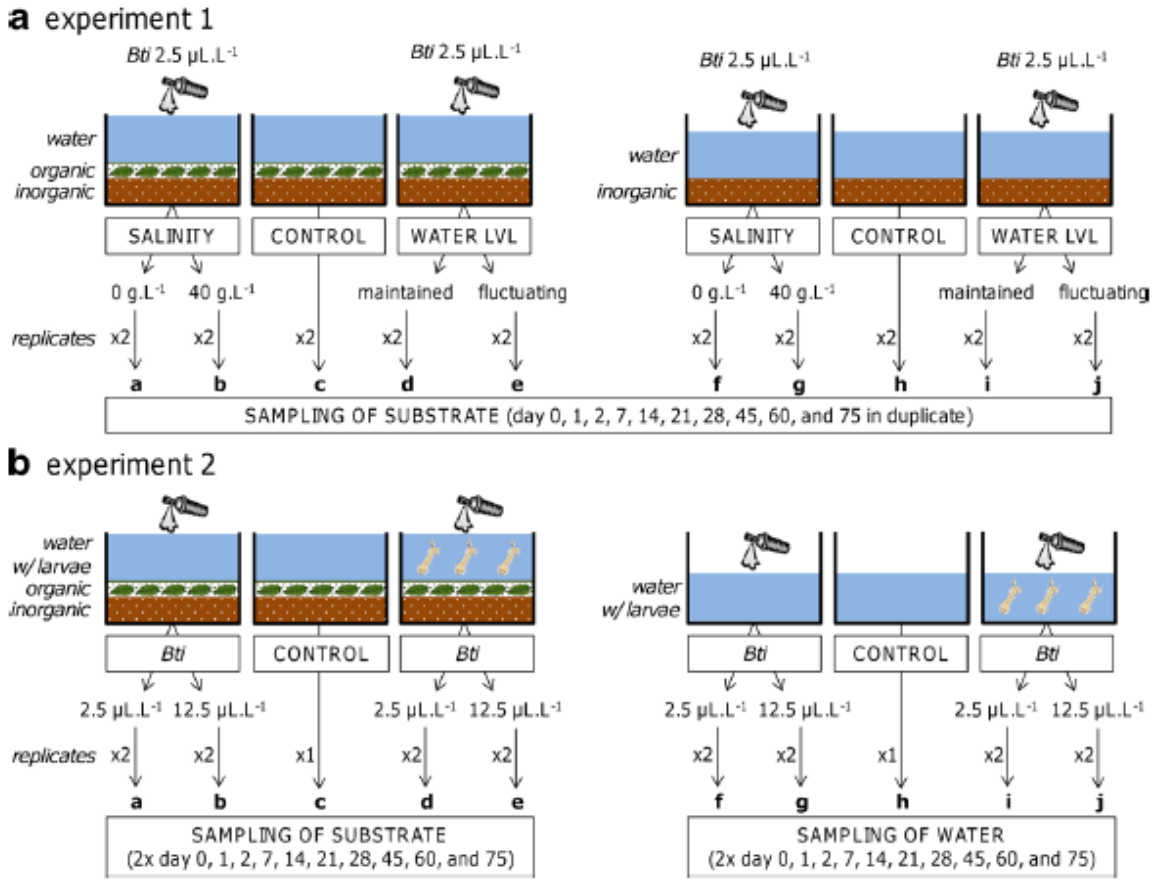

Fig. 2. Illustration of the experimental design of microcosms for evaluating the influence of **(A)** the presence of organic matter, the salinity (conditions a,b,f,g), the water level fluctuation (conditions d,e,i,j) and **(B)** the presence of larvae, with organic substrate (conditions a-e) or in water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

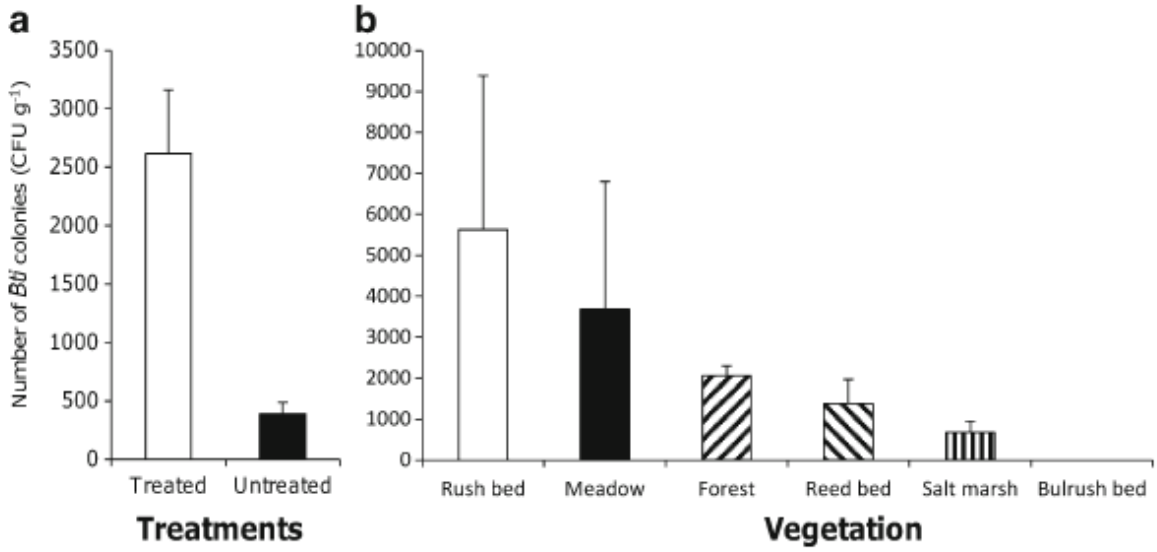

Fig. 3. Quantity of *Bti* spores (mean ± SE), expressed as colony forming unit (CFU) per gram of soil, in function of **A.** *Bti* treatment (Mann-Whitney test, $p < 0.001$) and **B.** Main vegetation type (Kruskall-Wallis test, $p < 0.001$)

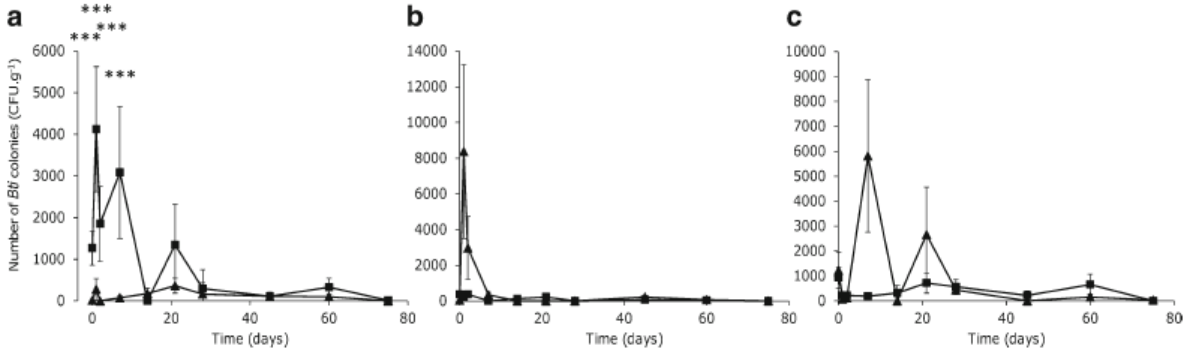

Fig. 4. Quantity of *Bti* spores (mean ± SE), expressed as colony forming unit per gram of soil, in function of three parameters (experiment 1 of microcosms). **A.** Influence of the substrate type on *Bti* abundance (inorganic, triangles; organic, squares). **B.** Influence of the salinity on *Bti* abundance (0 $g.L^{-1}$ of salt, triangles; 40 $g.L^{-1}$, squares). **C.** Influence of the water level on *Bti* abundance (fixed, triangles; fluctuating, squares). Mann-Whitney tests for each sampling date and for each modality: ***: $p < 0.001$.

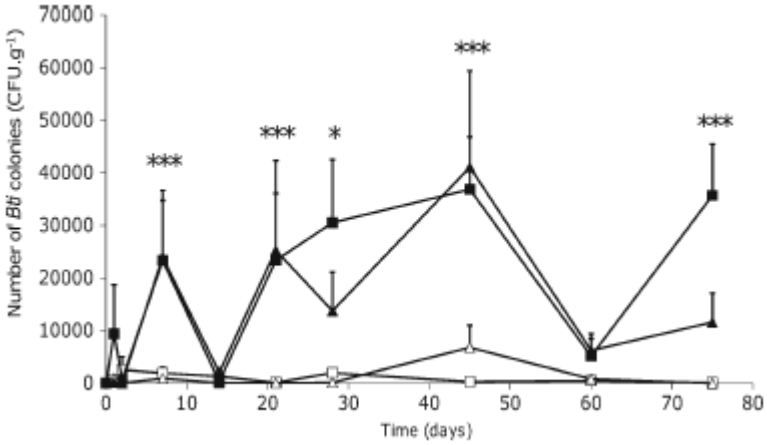

Fig. 5. Quantity of *Bti* spores (mean ± SE), expressed as colony forming unit per gram of substrate, in the microcosms treated with *Bti* at 2.5 (triangle) and 12.5 µL.L^{-1} (square), with (dark) or without (white) *Ae. aegypti* larvae (experiment 2 of microcosms). Kruskall-Wallis test: *: $p < 0.05$; ***: $p < 0.001$.