Scientific Reports

https://doi.org/10.1038/s41598-026-39702-1

Article in Press

Ecotoxicological and behavioural responses of aquatic model organisms to the MosChito raft bioinsecticide delivery system

Received: 3 November 2025

Accepted: 6 February 2026

Published online: 21 February 2026

Cite this article as: Negri A., Nigro L., Signorini S.G. *et al.* Ecotoxicological and behavioural responses of aquatic model organisms to the MosChito raft bioinsecticide delivery system. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-39702-1

Agata Negri, Lara Nigro, Silvia Giorgia Signorini, Silvia Locarno, Alberto Diana, Silvia Caccia, Paolo Gabrieli, Andrea Binelli, Luca Del Giacco, Claudio Bandi, Camilla Della Torre & Sara Epis

Ecotoxicological and behavioural responses of aquatic model organisms to the MosChito raft bioinsecticide delivery system

Agata Negri [a], Lara Nigro [b], Silvia Giorgia Signorini [a], Silvia Locarno [c,d], Alberto Diana [a], Silvia Caccia [a], Paolo Gabrieli [a], Andrea Binelli [a], Luca Del Giacco [a], Claudio Bandi [a], Camilla Della Torre [a], Sara Epis [a*]

[a] University of Milan, Department of Biosciences, Via Celoria 26, 20133 Milan, Italy

[b] University of Milano- Bicocca, DISAT – Department of Environmental and Earth Sciences, Piazza della Scienza 1 and 4, I-20126 Milan, Italy

[c] University of Milan, Department of Physics, Via Celoria 16, 20133 Milan, Italy, Milan, Italy

[d] Istituto Nazionale di Fisica Nucleare (INFN), via Celoria 16, 20133 Milan, Italy

* Corresponding author: sara.epis@unimi.it

Abstract

MosChito raft, a *Bacillus thuringiensis* (*Bti*)-based formulation, represent a promising device for mosquito larvae control in domestic environments. Its chitosan matrix and active component, the commercial *Bti* product VectoBac 12AS, ensure high specificity and minimal environmental impact.
This study evaluates the ecosafety of MosChito rafts by assessing their effects on two aquatic model organisms, *Daphnia magna* and *Danio rerio* embryos. Both organisms were exposed to different MosChito rafts (Matrix, Yeast, *Bti, Bti*+Yeast). Embryos and larvae of *D. rerio* were monitored to assess acute toxicity, changes in swimming behavior, and alterations in key biochemical markers (ROS and AChE). Daphnids were exposed from hatching to 14 days, and the same markers as in *D. rerio* were evaluated, as well as their fertility. Across all tests, MosChito raft showed no acute toxicity. Behavioral assays in *D. magna* revealed a slight but statistically significant reduction in swimming activity after exposure to specific formulations. Biochemical analyses revealed mild oxidative stress in *D. magna*, although cholinergic signaling remained unaffected. No significant impact on *D. magna* fertility was found. Overall, our findings suggest that MosChito raft exerts negligible toxic effects on non-target aquatic species, supporting its use as an environmentally safe mosquito control tool, consistent with integrated pest management strategies aimed at minimizing ecological impact.

Key words

Mosquito larval control; vector management, *Bacillus thuringiensis* subsp. *israelensis* (*Bti*); *Daphnia magna*; *Danio rerio*; ecotoxicology

Introduction

Interest and concern regarding the eco-toxicological impact of insecticide products have been steadily increasing in recent years. This trend is driven by research that has heightened awareness of the ecological effects associated with these products, increased public attention and social pressure, as consumers are becoming more informed about health and environmental risks, and stringent regulations imposed by regulatory agencies for environmental safety (US Environmental Protection Agency - EPA; European Regulation of biocidal products – BPR) [1]. Such concerns are particularly relevant for products requiring frequent use in urban settings, where they are in close contact with humans and domestic animals [2,3].
Among these products are those used for mosquito control, which differ in nature and application methods depending on the targeted developmental stage. Chemical and biological products aimed to control the immature stages of these insects, which live in confined aquatic environments, typically exert a more direct and immediate effect on the target, compared to those acting on the adults [4]. However, depending on the environment of application, high localized doses at the point of application may damage harmless species. Although these products remain a crucial component of integrated mosquito control management in temperate zones [5,6], they must comply with regulations (EU, 2012; Regulation 528/12), which limit the use of chemical products in favor of bioinsecticides, mainly of microbial origin.

Bacillus thuringiensis-based products are used in the control of a wide range of insect crop pests and vectors and hold a dominant position in the biopesticide sector, as evidenced by data showing that *Bt*-based biopesticides account for approximately 53% of the global biopesticide market (Centre for Agriculture and Bioscience International (CABI 2010)) [7,8]. More generally, entomopathogenic bacteria (EPB) represents 66% of the global microbial pesticide market, with *Bt* varieties representing over 90% of this category [8]. As Ragasruthi's review (2024) [9] points out, *Bt*-based biopesticides offer specificity, environmental friendliness, and cost-effectiveness in pest management, representing a vital area of research that should focus on the development of advanced formulations to meet the regulatory challenges of the time.
Among these, the bacterial entomopathogen *Bacillus thuringiensis* var. *israelensis* (hereafter *Bti*) represents the most widely used biocontrol method for mosquito larvae [10]. Its selective action on larvae (due to specific midgut luminal pH and proteases that favors solubilization and activation of *Bti* toxins, and to the presence of toxin-specific receptors on brush border membranes of the midgut epithelium) and its low environmental persistence have promoted the production of various formulations, such as liquids, water-dispersible granules, powders, and pellets, and its use in different contexts worldwide [11,12].
Regarding *Bti*, recent research has highlighted its low potential for inducing resistance in mosquitoes, due to the different targets of the Cry toxins included, and the synergic mechanism of action of Cry and Cyt toxins [13,14], while studies on non-target species (NTOs) indicate a very limited direct impact (evidence in Chironomids) or no impact [15]. Its low environmental impact is attributed to its negligible residual effect, due to rapid degradation, particularly in the presence of organic substances that bind and denature the toxins [16,17]. Nevertheless, the necessary repeated applications may have multiple effects, such as an increase in selective pressure and a decrease in insect susceptibility to *Bti*-based products [18].

As a solution to this issue, chemical or natural additives are often included to a wide range of formulations to enhance their efficacy and persistence. However, the toxic effects of these new compounds on the environments where they are released remain largely unknown [19]. A more effective approach would involve the use of a constant and controlled release system, delivering high insecticidal doses to mosquito larvae, without spreading *Bti* formulate into the environment. This has been achieved through the development of a new device, the MosChito raft: a tablet composed of a chitosan hydrogel crosslinked with genipin, which is highly porous, thus ensuring good buoyancy, and is dark blue, thus making it more attractive to mosquito larvae seeking shelter [20,21].

The effectiveness of MosChito rafts lies in their design, which encapsulates the insecticide and releases it only through direct erosion and ingestion by the target organism, rather than through diffusion into the water [21,22]. The product includes the commercial product *Bti*-based VectoBac 12AS, as well as cells of the common yeast *Saccharomyces cerevisiae*, used as an attractive food source for mosquito larvae [23]. The efficacy of this product has been tested in previous studies on larvae of different mosquito species, both native (*Culex pipiens*) and invasive (*Aedes albopictus* and *Aedes koreicus*) [21,24,25]. The encouraging results obtained under laboratory and semi-field conditions suggest that the MosChito rafts may be suitable for future field trials, but only after assessing their toxicity to other susceptible and non-target species.

The aim of this study was to evaluate the ecotoxicological effects of MosChito rafts using two aquatic model organisms: *Danio rerio*, at the embryonic and larval stages (up to 120 hours post-fertilization-hpf), and *Daphnia magna*, from the neonatal stage through 14 days of development. While previous studies [21] have demonstrated that the *Bti* larvicidal commercial product does not exert toxic effects in the absence of direct contact or ingestion by mosquito larvae, thanks to negligible release into the surrounding water, it remains uncertain whether the same release remains negligible also for other organisms.
Accordingly, the present study aimed to assess the potential effects of MosChito rafts on two widely used aquatic model organisms employed in ecotoxicological testing. The evaluation was conducted through survival tests, motility assays, biomarkers of oxidative stress and neurotoxicity, and fertility assays in *D. magna*.

Results

Toxicity effects on zebrafish embryos

Embryotoxicity

Following a 120-hour exposure, we observed no mortality across all treatment groups (Matrix, Yeast, *Bti*, *Bti*+Yeast), with survival rates in each experimental group exceeding 91%. Mortality was higher during the initial 24 hours for all groups, after which the survival rate remained stable throughout the experiment (Log-rank (Mantel-Cox) test, df= 4 $P = 0.3333$) (**Supplementary Figure 1**).

Effects on swimming behavior

Fig. 1A and 1B reports the results on swimming behavior of the embryos. No significant differences between treatments or between treatments and controls have been observed, both in terms of distance traveled (**Fig. 1A**) (ANOVA, $F(4,226)= 0.8399$, $P = 0.5011$) and percentage of movement (i.e., the average percentage of time - frames - the animal was deemed mobile across the trial) (**Fig. 1B**) (ANOVA, $F(4,232)= 1.041$, $P = 0.3868$). The results suggest no impact of the rafts on the animals' locomotor capacity.

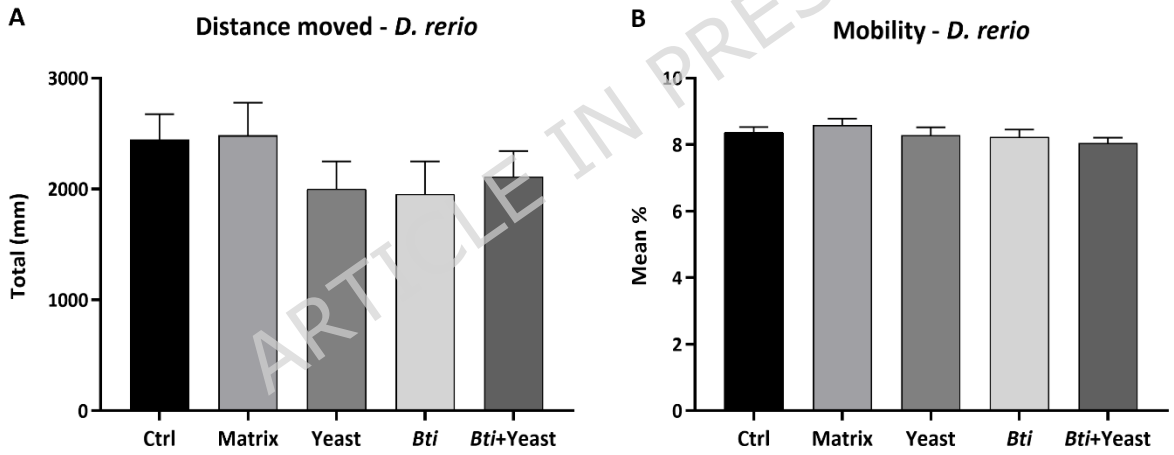

Fig. 1. Swimming behavior of zebrafish larvae following a 120-hour exposure to various MosChito raft compositions. **(A)** Total distance moved measured as total mm and **(B)** mobility measured as mean (%). Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeasts (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Values represent the mean ± standard error (SE) of three independent biological replicates.

Effects on biomarkers

Fig. 2A and **2B** show ROS levels and AChE activity in zebrafish, respectively, selected as biochemical endpoints (biomarker) of oxidative stress and cholinergic neurotoxicity [26,27]. ROS levels showed no significant differences across groups (Kruskal–Wallis test, $H(4) = 6.58$, $P = 0.1598$), and the same was true for AChE activity (ANOVA, $F(4,25)= 1.893$, $P = 0.1431$). These results indicate that the observed variations fall within normal biological variability and are unlikely to have biologically relevant effects on animal welfare.

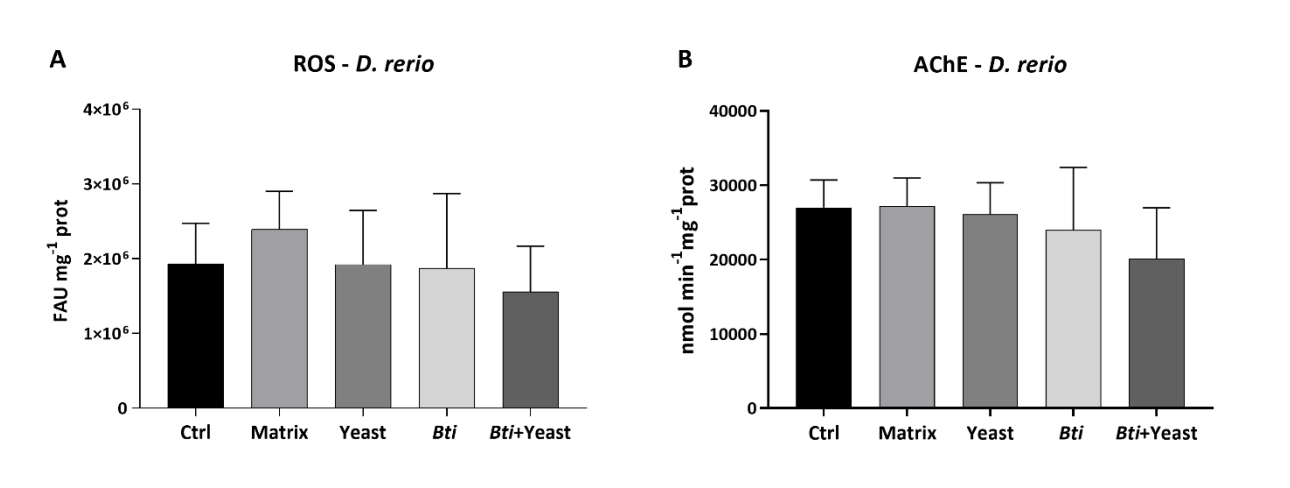

Fig. 2. Biomarkers in *D. rerio* zebrafish larvae after exposure to MosChito rafts. (A) Reactive oxygen species (ROS) levels, expressed as fluorescence arbitrary units (FAU) per mg of protein, and **(B)** acetylcholinesterase (AChE) activity, measured as nmol/min/mg of protein, are shown for each treatment group: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeasts (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Values represent the mean ± standard error (SE) of three independent biological experiments.

Toxicity effects on *Daphnia magna*

Acute toxicity

The assays evaluating the effects of MosChito rafts on the viability of neonatal *D. magna* were conducted using the same protocol as for zebrafish, except for the duration of exposure. Live and dead counts, up to day 14, indicated no significant differences in viability across all treatments (Log-rank (Mantel-Cox) test df = 4, P = 0,0885) (**Supplementary Figure 2**).

Behavioral effects

Two parameters were investigated to evaluate whether MosChito rafts influence the locomotor activity of *D. magna*. In the movement analysis, during alternating light and dark phases, a decrease in locomotor activity was observed in the group treated with the MosChito raft containing *Bti* and yeast compared to Ctrl. Specifically, the total distance traveled by this group was significantly lower than that of the control group, as illustrated in **Fig. 3A** (ANOVA, $F(4,324)= 2.726$, P = 0.0294; Tukey's multiple comparisons test, adjusted P = 0.0528 for Ctrl vs. *Bti*+Yeast). Additionally, the mean mobility percentage of Ctrl group also differed significantly with the Matrix alone and the *Bti*+Yeast group (ANOVA test, $F(4,324)= 3.564$, P = 0.0073; Tukey's multiple comparisons test, adjusted P = 0.0303 for Ctrl vs. *Bti*+Yeast and P = 0.0057 for Ctrl vs. Matrix) (**Fig. 3B**). These findings suggest that exposure to MosChito rafts influences very little the locomotor behavior of *D. magna*.

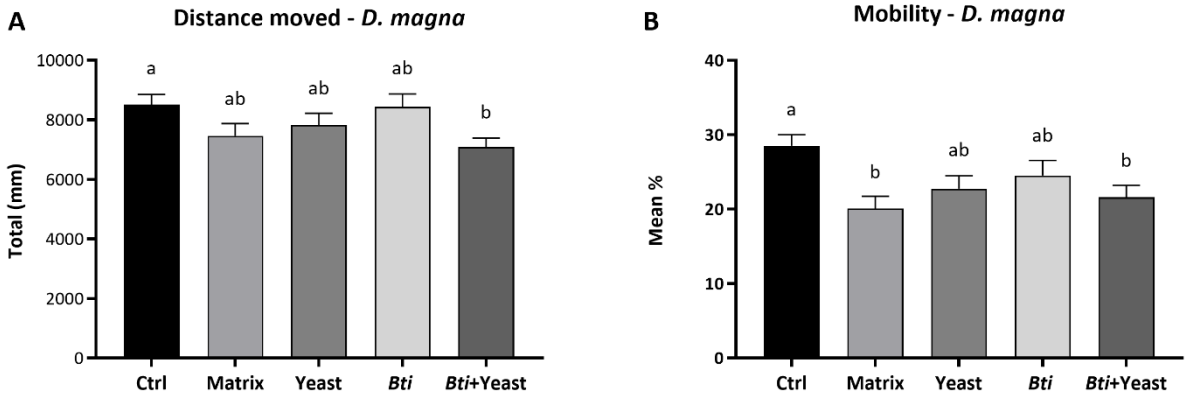

Fig. 3. Swimming behavior of *D. magna* following a 15-day exposure to MosChito rafts. **(A)** Total distance moved, measured as total mm. **(B)** Mobility, measured as average (%) of time spent in active movement. Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeast (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Data are presented as mean ± standard error (SE) of three independent biological experiments; different letters indicate statistically significant differences (**Supplementary Table 1**).

Effects on biomarkers

Fig. 4A and **4B** illustrate the results of the analyses on ROS levels and AChE activity, respectively. Regarding the production of ROS, a significant difference was observed between the Ctrl group and the group treated with *Bti*+Yeast (Ctrl vs *Bti*+Yeast P = 0.0468 with Tukey's multiple comparisons test after ANOVA, $F(4,25)= 2.961$, P = 0.0394). The statistical analysis for ROS was performed on log-transformed data [Y = log(Y)] to achieve normalization (**Supplementary Table 2**), whereas the graph (**Fig. 4A**) displays the raw values to allow a clearer representation of differences among groups. **Fig. 4B** shows no statistically significant differences in AChE activity (ANOVA, $F(4,25)= 1.344$, P = 0.2814).

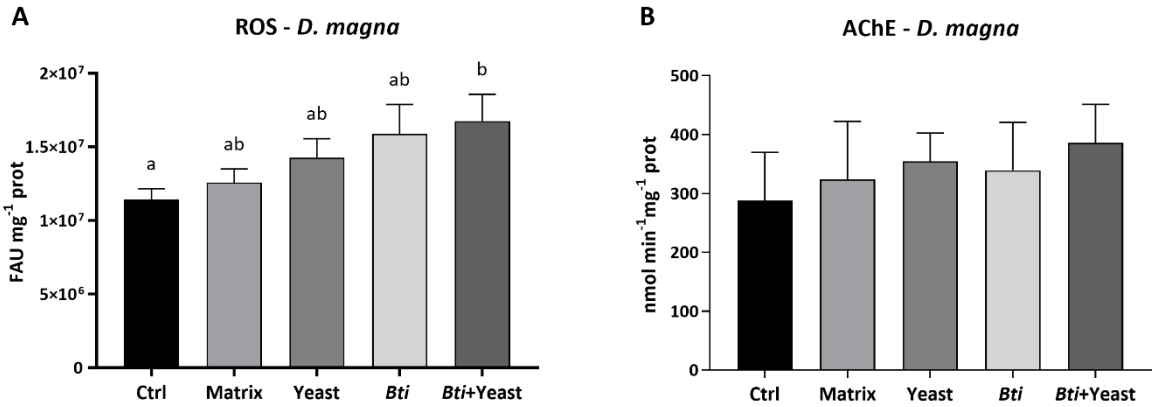

Fig. 4. Analysis of biomarkers in *D. magna* after exposure to MosChito rafts. (A) Reactive oxygen species (ROS) levels, expressed as fluorescence arbitrary units (FAU mg^{-1}protein). **(B)** Acetylcholinesterase (AChE) activity, expressed as nmol $min^{-1}mg^{-1}$ protein.

Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeast (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Data are presented as mean ± SE for each group of three independent biological experiments; different letters above the bars indicating statistically significant differences between groups

Reproduction during exposure

The effects of MosChito rafts on the reproductive capability of *D. magna* during the 14-day treatment period are illustrated in **Fig. 5**. The fertility tests were consistent with the typical reproductive peaks observed in *D. magna* cultures, starting on day 10 and continuing until day 14, when the experiment was concluded (**Fig. 5A**). All groups showed a comparable pattern of neonate production, with similar values throughout the treatment period. Although the *Bti*+Yeast group exhibited a slightly more pronounced peak on day 11 compared to the others, this variation was not statistically significant (Friedman's test for matched data per day, df=4, P = 0.1972), indicating that the observed differences fall within the range of biological variability.

The average number of neonates produced over the 14 days in the Ctrl group was 211.56 ± 9.58 (SE), a value comparable to those observed in the other treatment groups (Matrix = 236.11 ± 29.40; Yeast = 210 ± 24.29; *Bti* = 264.56 ± 16.68; *Bti*+Yeast = 276.89 ± 20.55) (**Fig. 5B**). These findings indicate that fecundity was not affected by the presence of MosChito rafts, regardless of their composition, in terms of both the total number of neonates and the timing of births (ANOVA, $F(4,40)= 2.621$, P = 0,05).

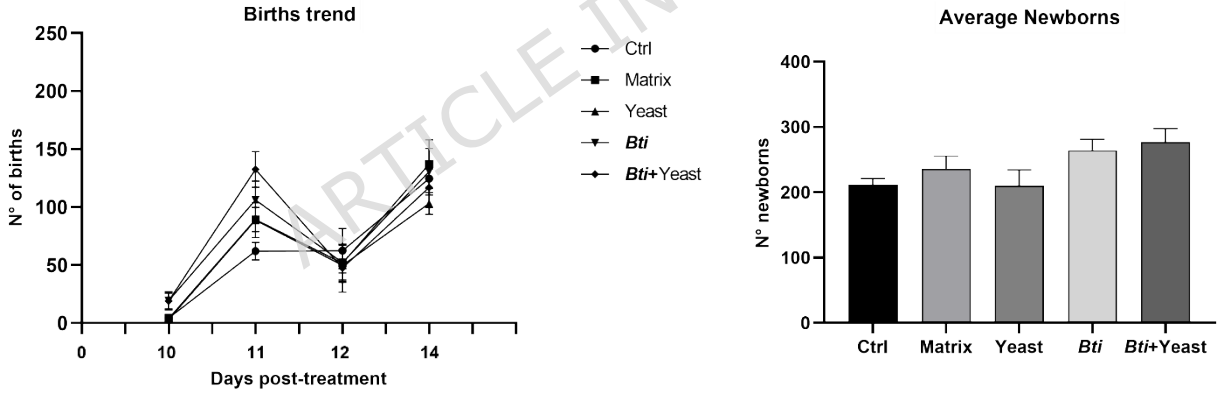

Fig. 5. Impact of MosChito rafts on the reproductive capacity of *D. magna* over 14 days. (A) Daily mean of neonates across the 14-day period. **(B)** Average number of neonates produced per treatment group over the entire 14-day period. Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeast (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). The Data are presented as mean ± SE of three independent experiments.

Discussion

This study aimed to evaluate the ecotoxicological effects of MosChito rafts, a novel mosquito control device, on two aquatic model organisms, *D. rerio* and *D. magna.*

The results of this study offer valuable insights into the environmental safety profile of this product, demonstrating that this mosquito control method, whose efficacy had been

determined in previous studies [21,24,25], has a negligible impact on these two non-target aquatic species. Contrary to all other commercial products that are dissolved in water, MosChito raft achieves acute toxicity on larvae without any release into the water but rather direct ingestion. The product's maximum functionality, and thus its potential toxicity, is lost once it is no longer confined within the hydrogel matrix.
Embedding the bioinsecticide within a protective matrix is also useful to prevent potential interactions that, regardless of whether they cause harm, could remove bioinsecticide from the environment, ultimately reducing its effectiveness. For instance, competition for food uptake by *Daphnia curvirostris* has been shown to decrease mosquito larval mortality following the application of *Bti* simply dissolved in water [28].

Regarding the two model species, it should be noted that acceptable toxicity values for zebrafish and *D. magna* vary depending on the substance and the specific endpoint being measured (in this study we measured mortality, motility and developmental effects. In general, for sub-chronic toxicity assays such as those conducted in this study, *D. magna* is considered more sensitive than zebrafish, exhibiting toxic effects at lower concentrations [29,30]. On the other hand, being a Vertebrate, zebrafish show high homology with mammals, thus representing a more powerful model to mimic potential toxic effects on humans.
Regarding the biolarvicide, *Bti* is among the safest mosquito control agents currently available, consistently shown to cause no significant harm to non-target organisms such as *D. rerio*. This contrasts sharply with the documented toxicity of many conventional insecticides used against both larval and adult mosquitoes [31,32].
The absence of acute in toxicity zebrafish embryos detected across all treatment groups align with previous studies that have demonstrated the low toxicity of *Bti*-based products to non-target aquatic organisms, including fish species [12,17,33]. The absence of significant differences in survival between treatments and controls suggests that the raft matrix, as well as its individual components (yeast and *Bti*), do not exert harmful effects on zebrafish embryos, a critical life stage known to be sensitive to environmental contaminants [34].
Behavioral assays supported these findings. MosChito rafts does not impair nervous system function or motor coordination in zebrafish embryos. These results align with previous studies on *Bti* dissolved in water, which consistently report a low neurotoxic potential in non-target species [35]. While Grisolia et al. (2009) [36] reported developmental delay and genotoxicity in zebrafish embryos and adults exposed to purified δ-endotoxins of *Bti* at concentrations exceeding 50 mg/L, more recent data from Padilha et al. (2024) [35] questioned these findings, demonstrating negligible toxicity at lower exposure levels (10 mg/L). Interestingly, Padilha et al. observed only one motor-related effect, a reduction in larval body length, which may indirectly impact locomotion and reflect an anti-nutritional mechanism. Similarly, Gonçalves et al. (2023) [37] reported that *Bti* protoxins induced potential anti-nutritional effects in adult zebrafish, which could plausibly explain the reduced larval size observed in earlier developmental stages.
In this context, it would be valuable to further investigate the possible impact of MosChito rafts on larval growth and adult phenotypes beyond 120 hpf. However, it is important to consider that, in our experimental design, the actual exposure to *Bti* was minimal despite the relatively high nominal concentration (97 mg/L), due to the localized and minimal release of the active ingredient from the raft matrix. Consequently, the

internal dose experienced by zebrafish embryos may be negligible compared to nominal concentration, which may explain the absence of neurotoxic or developmental effects even under conditions simulating worst-case exposure.
Biochemical biomarker analyses provided additional evidence of the rafts' environmental compatibility. Exposure to MosChito rafts does not induce oxidative stress or disrupt cholinergic signaling in zebrafish.
In *D. rerio*, *Bt* or *Bti* does not generally induce oxidative stress when tested using commercial formulations or environmentally relevant exposure scenarios. Available studies report no significant changes in ROS levels or other oxidative stress–related biomarkers in zebrafish embryos or larvae exposed to *Bti*-based products, together with an absence of developmental or behavioral alterations [35, 36]. Moreover, evidence of oxidative stress or molecular damage in zebrafish has been reported only under non-representative experimental conditions, specifically following exposure to purified *Bt* δ-endotoxins at very high concentrations. These exposure scenarios are not comparable to commercial formulations or realistic field applications and differ substantially from those typically adopted in ecotoxicological risk assessment [36].

Similarly, no toxicity was observed in *D. magna*, with survival rates above 90% in all treatments. Although *D. magna* is more sensitive than zebrafish in acute ecotoxicological tests, as described above, in this case, MosChito raft did not induce a harmful impact on this species. These results agree with those reported by Duchet et al. (2011) [38], who found that *Bti* had no adverse effects on population survival or growth of *D. magna* under both laboratory and field mesocosm conditions. Additionally, as already emphasized, in our study, the commercial product VectoBac 12AS is incorporated within the matrix rather than directly dissolved in water, minimizing the potential ingestion of *Bti* (see also Pitton et al., 2023) [21]. We propose that the slight mortality observed in our study, during the initial days of exposure, is more likely due to natural variability rather than a direct toxic effect of the rafts. Notably, the only factor that may have compromised individual survival was the adhesion of the raft matrix to the antennae of some specimens (see **Supplementary Figure 3**), a characteristic of the formulation that warrants further optimization in future developments.

Behavioral assays in *D. magna* revealed a slight reduction in swimming activity in the *Bti*+Yeast and Matrix groups compared to Ctrl groups. However, the magnitude of these changes was minimal and is unlikely to reflect any biologically relevant or ecologically harmful impairment. These results do not appear to be related to the previously mentioned issue of matrix adhesiveness to the antennae, as the observed effect was present in immature stages rather than in adults, on which the swimming activity test was conducted. Moreover, the fact that reduced activity was also recorded in individuals exposed to the Matrix treatment, but not in those treated with *Bti* alone, excludes bioinsecticide as a potential neurotoxic agent causing locomotor impairment. Regarding the consequence of *Bti*, to date, there are studies that have showed no toxicity on the developmental capacities of aquatic invertebrates, among which mollusks, crustaceans (including Cladocera, Amphipoda, and Copepoda), and insects (including Ephemeroptera) or vertebrates, such as fish and amphibians [17,39,40]. Conversely, a more recent study reported a dose-dependent effect of *Bt* Cry1Ab and Cry2Aa toxins on the development of *D. magna*. According to Bøhn et al. (2016) [41], *D. magna* specimens exposed to high concentrations of Cry toxins (4.5 mg/L) exhibited reduced body size,

while those exposed to lower concentrations (0.75 mg/L) showed increased growth compared to controls. These alterations in body size could potentially influence the organisms' swimming performance and mobility. Additionally, the review by Land and colleagues (2023) [12] highlights potential indirect effects of *Bti* treatments on Crustacea and Chironomidae, suggesting possible disruptions to food webs and ecosystem dynamics. However, the same authors also acknowledge that these conclusions are based on a limited number of studies, emphasizing that this field remains underexplored. In the case of MosChito rafts, the negligible release of *Bti* doses [21] makes any toxic effects of the bioinsecticide on *D. magna* unlikely. Furthermore, at the end of the tests, no significant differences in body size were observed between treatment groups. Nevertheless, further investigation is needed to determine whether the observed decrease in mobility, caused by the combined presence of all MosChito raft components, could influence ecological interactions, such as predator avoidance or foraging behavior.

Regarding the biochemical analyses, AChE activity and ROS levels were selected as the primary endpoints to assess neurotoxicity and oxidative stress in non-target organisms. In addition to these biochemical parameters, behavioral and motility assays were included as complementary indicators, providing a functional link between biochemical alterations and organism-level effects. In particular, our study revealed a significant increase in reactive ROS levels in *D. magna* exposed to the *Bti*+Yeast treatment, suggesting a mild oxidative stress response. However, AChE activity remained unchanged across treatments, indicating no disruption of cholinergic signaling.
In Chen et al. (2018) [42], no alterations in antioxidant enzyme activity of *D. magna*, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), were observed in the presence of the *Bt's* protein Cry1C, although the protein was detected in the bodies of exposed individuals, confirming filtration and exposure, it did not result in evident lethal or sublethal effects. Consistently, the biochemical responses observed in the present study were limited to the evaluated endpoints and did not indicate biologically relevant alterations under the tested conditions. A broader evaluation, incorporating additional biochemical and molecular parameters, such as other oxidative stress biomarkers and gene expression analyses, would allow a more comprehensive characterization of potential sublethal effects and their underlying mechanisms.

Finally, reproductive assays demonstrated that MosChito rafts did not significantly affect the fecundity of *D. magna*. The average number of offspring produced in treated groups was comparable to that in controls, and birth trends followed the typical reproductive cycle of this species [43]. These results highlight the safety of MosChito raft in terms of its impact on population dynamics in *D. magna*. Notably, the *Bti*+Yeast group exhibited a slightly higher number of offspring, although this difference was not statistically significant. Previous studies have suggested that yeast, as a nutrient source, may enhance reproductive output in *D. magna* [44]. While this effect was not pronounced in the present study, it warrants further exploration under varying environmental conditions and nutrient availability.

Although the application of MosChito rafts in urban environments and within anthropogenic artificial containers significantly limits exposure to non-target organisms, it remains essential to conduct further testing on species that are naturally occurring in urban ecosystems and may come into incidental contact with the rafts.

Conclusion: implications for mosquito control and future perspectives

MosChito raft emerges as a promising alternative to conventional mosquito control, as its *Bti*-based selective action and controlled larval-ingestion release minimize environmental dispersion and risks against non-target. Compared to chemical insecticides, which often pose significant risks to aquatic ecosystems, biopesticides like *Bti* have a well-documented history of safety, while having good efficacy [10,45]. Moreover, in future developments, the incorporation of engineered yeasts expressing small RNAs, might increase the specificity of MosChito raft to mosquito larvae, for example, through the targeting of mosquito defense mechanisms.

Future studies should prioritize field-based assessments to validate laboratory findings, evaluate long-term environmental effects across aquatic habitats, and examine impacts on additional non-target species, including optimization of formulation parameters (e.g. insecticide concentration) for application under natural conditions.

Materials and methods

Formulation and composition of MosChito rafts

To assess the ecotoxicity of MosChito rafts, different formulations were prepared to evaluate the potential impact of each individual component on the model organisms. According to protocols established in our previous studies [21,22,24], the MosChito raft matrix consists of chitosan chemically crosslinked with genipin, forming a mechanically stable, insoluble, and biodegradable polymer network capable of entrapping the *Bti* formulation. The matrix was characterized using physicochemical, structural, and functional assays to assess crosslinking efficiency, stability in water, controlled bioavailability of the active ingredient, and larvicidal performance [22]. MosChito rafts were produced in four formulations: empty rafts (Matrix), rafts containing only *Saccharomyces cerevisiae* (strain SY2080; 10^7 cells/raft) (Yeast), rafts containing only the *Bti*-based insecticide VectoBac 12AS (420 µl ml^{-1} final concentration) (*Bti*), and rafts containing both yeast and VectoBac 12AS (*Bti*+Yeast).

Ecotoxicological assays

Zebrafish husbandry

Wild type adult zebrafish of the AB-TL line were bred at the Department of Biosciences of the University of Milan (authorization issued by the municipality of Milan PG 198283/2019). Fish were reared in flow-through conditions at a water temperature between 28 °C and 28.5 °C, with a photoperiod of 14 hours of light and 10 hours of darkness and fed three times a day with small granular food (ZM Fish Food & Equipment).

Zebrafish embryotoxicity test

To achieve better robustness of the exposure results, embryos were obtained from different pairs of adults. For the exposure experiment, embryos were sorted and used for exposure within three hours from fertilization. The assay was carried out according to the OECD procedures (TG 236, OECD 2013) with some necessary modifications. In compliance with the National law on welfare of animals subject to scientific purposes (Legislative Decree No. 26/2014), exposure ended within 120 hours post fertilization (hpf).

Differently from the OECD protocol, the test was carried out using 25 normally developed embryos placed in beakers (500 mL), each one representing an experimental group. The use of 500 mL beakers as test chamber was justified by the fact that the MosChito raft is a tablet with a diameter around 1.5 cm and a thickness around 5 mm. For this reason, it was not possible to execute the assay in a 24-well plates as indicated by the protocol, neither in beakers with small diameters. Moreover, this exposure is more representative of realistic application conditions, where one raft is supposed to be administered in approximately 1 L of water. Since also the control group was reared in the same test chamber, we assume that this modification should not affect the reliability of the test. Exposures were run in an incubator at a constant temperature of 27 °C.

Three independent exposure experiments were carried out with two technical replicates for each one. Every 24 hours, embryos were observed under the stereomicroscope to assess the lethal parameters: coagulated embryo, lack of somite formation, no detachment of the tail and lack of heartbeat. Moreover, some teratogenic parameters were evaluated: not completed epiboly, abnormal development of eyes, lack of spontaneous movement, edemas, reduced pigmentation, malformations, scoliosis and delayed growth, according to Schiwy et al. (2015) [46]. At the end of the exposure

period, twelve normally developed embryos from each experimental group were analyzed for their behavior. The remaining embryos were collected and frozen at -80 °C for the measurement of reactive oxygen species (ROS) content and acetylcholinesterase (AChE) activity.

Daphnia magna rearing

For the *D. magna* exposures, we used specimens (daphnids, younger than 24 hours) obtained from Daphtoxkit F (MicroBioTests, Inc.) ephippia. To hatch the ephippia, 2 L of standard freshwater were prepared (following UNI EN ISO 6341, 2013) by dissolving sodium bicarbonate ($NaHCO_3$; 129.5 mg), magnesium sulfate heptahydrate ($MgSO_4 \times 7H_2O$; 264.5 mg), calcium chloride dihydrate ($CaCl_2 \times 2H_2O$; 588 mg), and potassium chloride (KCl; 11.5 mg). The solution was aerated 15 minutes before using it. The ephippia were then placed in a micro-sieve, rinsed with tap water to remove the storage medium, and transferred to a Petri dish containing 15 mL of pre-aerated standard water. Incubation was carried out at 20°C under continuous illumination (6000 lx) for 72 hours. Before starting the test, the daphnids were fed for two hours with a suspension of the blue-green alga *Spirulina* spp..

Daphnia magna exposure

The exposure lasted 14 days under semi-static conditions at 20°C, with a light/dark cycle of 16 hours of light (1500 lx) and 8 hours of darkness, following the OECD Guideline 211 with slight modifications. For every experimental group (the same groups used for the *D. rerio* experiment) 50 individuals (< 24 hours), were placed in five 500 mL beakers, each corresponding to a different experimental group. Exposures were conducted in triplicate, with daily reports of viability and immobilization. Exposure assays were performed in three independent biological replicates. Water renewal was performed every 48 h according to the OECD protocol (OECD 211).

Throughout the exposure period, organisms were fed daily with a suspension of the green alga *Raphidocelis subcapitata*, according to the OECD protocol. The initial feeding concentration was 8×10^6 cells per individual per day until the daphnids reached 8 days of age, after which it was increased to 16×10^6 cells per individual per day [47]. Following the behavioral assessments, the same specimens were frozen at -80°C for the measurement of ROS content and AChE activity.

Swimming behavior

Zebrafish embryos were placed in 24-well plates and swimming behavior was assessed using the DanioVision observation chamber (Noldus Inc., Wageningen, The Netherlands). The swimming performances were measured following a light/dark transition locomotor response test developed for zebrafish embryos according to Leuthold et al. (2019) [48].

Concerning *Daphnia*, the protocol for analyzing swimming performance included a 10-minute acclimation period followed by two light (4400 lx)/dark cycles, each lasting 10 minutes, for a total duration of 30 minutes. Swimming behavior was assessed at the end of the exposure for 24 individuals per treatment.

Video data for both species were recorded at a sample rate of 30 frames/second via a high-speed infrared camera. The EthoVision XT software (Noldus Inc., Wageningen, The Netherlands) was used to analyze the total distance moved by each individual and the mobility %. The latter is the mean proportion of time the animal is classified as mobile, based on frame-by-frame area changes and predefined thresholds

Biomarker analyses

Biomarkers were measured in pooled embryos and daphnids from each experimental group. Samples were homogenized in 100 mM phosphate buffer at pH 7.4, 100 mM KCl, 1 mM EDTA, previously added with 100 mM dithiothreitol and protease inhibitor cocktail (1:100 v/v) (Merck KGaA), added at a ratio 1 embryo/10 µL. The homogenates were

centrifuged for 10 min at 15,000 ×g at 4 °C and the supernatant was used for the assays. The protein content was measured according to the Bradford method (1976) [49], calibrated on a standard BSA curve (0.1-0.5 mg mL^{-1}). The ROS content was measured using dichlorofluorescein diacetate (DCFH-DA) as substrate, according to the method described in Della Torre et al. (2018) [50]. The fluorescence was measured at 485/530 nm (excitation/emission) wavelengths using the EnSight Multimode Plate Reader (PerkinElmer). Data were expressed as measured fluorescence units (FU)·mg $proteins^{-1}$. For statistical analysis of *D. magna* ROS production, data were log-transformed [Log(Y)] to achieve normalization; however, untransformed values are presented in Results.

The AChE activity was measured according to the method of Ellman et al., (1961) [51] in a reaction mixture consisting of a potassium phosphate buffer (100 mM, pH 7.4), acetylthiocholine chloride (1 mM) and 5,5′ dithiobis-2-nitrobenzoic acid (0.5 mM). The reaction was run for 10 min and the absorbance was measured at λ = 412 nm. The activity was expressed as nmol $min^{-1}mg^{-1}$protein.

Assays were performed in three independent biological replicates.

Statistical analysis

All data obtained in this study were analyzed and graphically represented using GraphPad Prism software. Specifically, survival data for *both D. rerio* and *D. magna* were analyzed using the Log-rank (Mantel-Cox) test to perform a comparison of survival curves across different treatment groups. Only the most relevant statistical values are reported in the Results section, while the corresponding survival plots are not shown.

Significance of variations in swimming behavior, biomarker response and *Daphnia* fertility test were assessed using one-way ANOVA followed by Tukey's test, except for ROS levels in *D. rerio*, for which the data distribution required the use of the non-parametric Kruskal-Wallis test. Temporal trends in daily reproduction were examined using the Friedman test for matched data.

Ethical approval statement

The study involved mosquito larvae and *Daphnia* spp., which are invertebrates and therefore not subject to ethical approval requirements under applicable legislation.

Experiments involving zebrafish (*D. rerio*) were performed exclusively on embryos and larvae. No specimen included in this study exceeded 120 h post-fertilization (hpf). In accordance with European Directive 2010/63/EU, no specific ethical approval was required for the experiments conducted.

Accordingly, ethical approval from an institutional ethics committee or licensing body was not required for any of the experimental methods described in this manuscript.

Acknowledgements and Funding Information.
We thank Cristina Lenardi (University of Milan, Department of Physics) for her constructive suggestions. Study partially supported by UNIMI GSA-IDEA Project and network.

Funding was provided by the MUSA (Multilayered Urban Sustainability Action) project, funded by the European Union–NextGenerationEU under the National Recovery and Resilience Plan (NRRP) Mission 4 Component 2 Investment Line 1.5: Strengthening of research structures and creation of R&D “innovation ecosystems” and “territorial leaders in R&D”; PNRR project PE-13 INF-ACT “One Health Basic and Translational Research Actions addressing Unmet Needs on Emerging Infectious Diseases”

ADDITIONAL INFORMATIONS:

Declaration of Competing Interest
The authors declare no conflicts of interest regarding the manuscript entitled “Ecotoxicological and behavioural responses of aquatic model organisms to the MosChito raft bioinsecticide delivery system”.

Data Availability
Data will be made available on request from the corresponding author, S.E.

Ethics Declarations
All experiments involving *Danio rerio* and *Daphnia magna* complied with Directive 2010/63/EU and applicable national regulations. Work on D. rerio was limited to embryos up to 120 hpf, which are not classified as protected animals under the Directive. D. magna is an invertebrate species and therefore falls outside the requirement for ethical approval.

References

1. Lee, B. M. et al. Biocidal products: Opportunities in risk assessment, management, and communication. *Risk Analysis*, **44**(2), 493-507. (2024). https://doi.org/10.1111/risa.14160.

2. Bean, T. G. et al. Toxicological effects assessment for wildlife in the 21st century: Review of current methods and recommendations for a path forward. *Integrated environmental assessment and management*, **20**(3), 699–724. (2024). https://doi.org/10.1002/ieam.4795.

3. Sinha, V., and Shrivastava, S. Cypermethrin: an emerging pollutant and its adverse effect on fish health and some preventive approach: a review. *Indian journal of microbiology*, 64(1), 48–58. (2024). https://doi.org/10.1007/s12088-023-01153-x.

4. CDC 2024. Larval Source Management and Other Vector Control Interventions. June 2024. https://www.cdc.gov/malaria/php/public-health-strategy/larval-management.html.

5. Marrama, R.L. and Schaffner, F. ECDC (European Centre for Disease Prevention and Control) guidelines for the surveillance of invasive mosquitoes in Europe. August 2012. Stockholm, Sweden, Europe. *Eurosurveillance*. (2012). https://doi.org/10.2900/61134.

6. Virgillito, C. et al. Evaluation of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* combination against *Culex pipiens* in highly vegetated ditches. *Journal of the American Mosquito Control Association*, **38**(1), 40–45. (2022). https://doi.org/10.2987/21-7024.

7. Ortiz, A. and Sansinenea, E. *Bacillus thuringiensis* based biopesticides for integrated crop management. 10.1016/B978-0-12-823355-9.00015-8. (2022).

8. Smagghe, F., Spooner-Hart, R., Chen, Z.H., Mak, M. Biological control of arthropod pests in protected cropping by employing entomopathogens: efficiency, production and safety. *Biological Control*. 186. 105337. 10.1016/j.biocontrol.2023.105337. (2023).

9. Ragasruthi, M. et al. *Bacillus thuringiensis* (*Bt*)-based biopesticide: navigating success, challenges, and future horizons in sustainable pest control. *The Science of the total environment*, **954**, 176594. (2024). https://doi.org/10.1016/j.scitotenv.2024.176594.

10. Brühl, C. A. et al. Environmental and socioeconomic effects of mosquito control in Europe using the biocide *Bacillus thuringiensis* subsp. *israelensis* (*Bti*). *The Science of the total environment*,**724**, 137800. (2020). https://doi.org/10.1016/j.scitotenv.2020.137800.

11. Melo, A. L., Soccol, V. T., and Soccol, C. R. *Bacillus thuringiensis*: mechanism of action, resistance, and new applications: a review. C*ritical reviews in biotechnology*, **36**(2), 317–326. (2016). https://doi.org/10.3109/07388551.2014.960793.

12. Land, M., Bundschuh, M., Hopkins, R. J., Poulin, B., & McKie, B. G. Effects of mosquito control using the microbial agent *Bacillus thuringiensis israelensis* (*Bti*) on

aquatic and terrestrial ecosystems: a systematic review. *Environmental evidence*, **12**(1), 26. (2023). https://doi.org/10.1186/s13750-023-00319-w.

13. Lacey, L. A. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control*. Journal of the American Mosquito Control Association*, **23**(sp2), 133-163. (2007).

14. Paris, M. et al. Persistence of *Bacillus thuringiensis israelensis* (*Bti*) in the environment induces resistance to multiple *Bti* toxins in mosquitoes. Pest management science, **67**(1), 122–128. (2011). https://doi.org/10.1002/ps.2046.

15. Vaughan, I.P., Newberry, C., Hall, D.J., Liggett, J.S., Ormerod, S.J. Evaluating large-scale effects of *Bacillus thuringiensis var. israelensis* on non-biting midges (Chironomidae) in a eutrophic urban lake. *Freshwater Biology,* **53**(10):2117–28. (2008).

16. Sheeran, W., and Fisher, S. W. The effects of agitation, sediment, and competition on the persistence and efficacy of *Bacillus thuringiensis var. israelensis* (*Bti*). *Ecotoxicology and environmental safety*, **24**(3), 338–346. (1992). https://doi.org/10.1016/0147-6513(92)90010-z.

17. Boisvert, M., and Boisvert, J. Effects of *Bacillus thuringiensis* var. *israelensis* on target and non-target organisms: a review of laboratory and field experiments. *Biocontrol science and Technology,* **10.5**: 517-561. (2000).

18. Tilquin, M., et al. Long-lasting persistence of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) in mosquito natural habitats. *PloS one*, **3**(10), e3432. (2008). https://doi.org/10.1371/journal.pone.0003432.

19. McKie, B.G., Taylor, A., Nilsson T., Frainer, A., Goedkoop, W. Ecological effects of mosquito control with *Bti*: evidence for shifts in the trophic structure of soil- and ground-based food webs. *Aquat Sci* ;**85**(2):47. (2023).

20. Hapairai, L. K et al. Lure-and-kill yeast interfering RNA larvicides targeting neural genes in the human disease vector mosquito *Aedes aegypti*. *Scientific reports*, **7**(1), 13223. (2017). https://doi.org/10.1038/s41598-017-13566-y.

21. Pitton, S. et al. MosChito rafts as effective and eco-friendly tool for the delivery of a *Bacillus thuringiensis*-based insecticide to *Aedes albopictus* larvae. *Scientific reports,* **13**(1), 3041. (2023). https://doi.org/10.1038/s41598-023-29501-3.

22. Piazzoni, M. et al. Biodegradable floating hydrogel baits as larvicide delivery systems against mosquitoes. *Soft Matter*, **18**(34), 6443-6452. (2022).

23. Steyn, A., Roets, F., & Botha, A. Yeasts associated with *Culex pipiens* and *Culex theileri* mosquito larvae and the effect of selected yeast strains on the ontogeny of *Culex pipiens*. *Microbial ecology*, **71**(3), 747–760. (2016). https://doi.org/10.1007/s00248-015-0709-1.

24. Negri, A. et al. MosChito rafts as a promising biocontrol tool against larvae of the common house mosquito, *Culex pipiens*. *PloS one*, **18**(12), e0295665. (2023). https://doi.org/10.1371/journal.pone.0295665.

25. Negri, A. et al. The biolarvicide *Bacillus thuringiensis* var. *israelensis* is effective against *Aedes koreicus*, either dissolved in water or delivered through eco-compatible chitosan-based hydrogels. *Current Research in Parasitology & Vector-Borne Diseases*, **6**, 100197. 10.1016/j.crpvbd.2024.100197. (2024).

26. Eriksson Wiklund, A. K., Adolfsson-Erici, M., Liewenborg, B., & Gorokhova, E. Sucralose induces biochemical responses in *Daphnia magna*. *PloS one*, **9**(4), e92771. (2014). https://doi.org/10.1371/journal.pone.0092771

27. Heredia-García, G. et al. Realistic concentrations of Bisphenol-A trigger a neurotoxic response in the brain of zebrafish: Oxidative stress, behavioral impairment, acetylcholinesterase inhibition, and gene expression disruption. *Chemosphere*, **330**, 138729. (2023). https://doi.org/10.1016/j.chemosphere.2023.138729

28. Becker, N. Community participation in the operational use of microbial control agents in mosquito control programs. *Bulletin of the Society for Vector Ecology* 17, 114-118. (1992).

29. Xiong, Y., Chen, X., Li, F., Chen, Z., & Qin, Z. Zebrafish larvae acute toxicity test: A promising alternative to the fish acute toxicity test. *Aquatic toxicology (Amsterdam, Netherlands)*,**246**, 106143. (2022). https://doi.org/10.1016/j.aquatox.2022.106143

30. Kerdoun, R., Kerdoun, M. A., Laid, T., Hakim, B. Sensitivity of *Daphnia magna*: acute toxicity evaluation of 22 metals. *Journal of Pharmacy and Nutrition Sciences*, **1,** 62-70; 10.29169/1927-5951.2024.14.08. (2024).

31. XingYu, L. et al. Developmental toxicity and neurotoxicity of synthetic organic insecticides in zebrafish (*Danio rerio*): A comparative study of deltamethrin, acephate, and thiamethoxam. *Chemosphere,* **199** : 16-25. (2018)

32. Blanc, M. et al. The insecticide permethrin induces transgenerational behavioral changes linked to transcriptomic and epigenetic alterations in zebrafish (*Danio rerio*). *The Science of the Total Environment:* **779**, 146404. (2021). https://doi.org/10.1016/j.scitotenv.2021.146404.

33. Lagadic, L. et al. No association between the use of *Bti* for mosquito control and the dynamics of non-target aquatic invertebrates in French coastal and continental wetlands. *The Science of the total environment*, **553**, 486–494. (2016). https://doi.org/10.1016/j.scitotenv.2016.02.096.

34. Scholz, S. et al. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. *Environmental Science and Pollution Research*, **15**(5), 394-404. (2008).

35. Padilha, R. M. O. et al. Assessment of toxicity of pyriproxyfen*, Bacillus thuringiensis*, and malathion and their mixtures used for mosquito control on embryo-larval development and behavior of zebrafish. *Environmental science and pollution research international*, **31**(30), 42672–42685. (2024). https://doi.org/10.1007/s11356-024-33955-x.

36. Grisolia, C. K. et al. Genotoxic evaluation of different delta-endotoxins from *Bacillus thuringiensis* on zebrafish adults and development in early life stages.

Mutation research, **672**(2), 119–123. (2009). https://doi.org/10.1016/j.mrgentox.2008.10.017.

37. Gonçalves, Í. F. S. et al. An innovative insecticidal approach based on plant protease inhibitor and *Bt* protoxins inhibits trypsin-like activity in zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*,**267**, 109576. (2023).

38. Duchet, C., et al. Chitobiase activity as an indicator of altered survival, growth and reproduction in *Daphnia pulex* and *Daphnia magna* (Crustacea: Cladocera) exposed to spinosad and diflubenzuron. *Ecotoxicology and environmental safety*, **74**, 800-10. 10.1016/j.ecoenv.2010.11.001. (2011).

39. Miura, T., R. M. Takahashi and F. S. Mulligan, III. Effects of the bacterial mosquito larvicide *Bacillus thuringiensis* serotype H-14 on selected aquatic organisms." Mosquito News, **40**, 4, pp. 619–22. (1980). https://www.biodiversitylibrary.org/part/131402.

40. Arshad, A. *Bacillus thuringiensis* serovar*. israelensis* (ABG-6108) against chironomids and some non target aquatic invertebrates. *Journal of Invertebrate Pathology,* **38.2**: 264-272. (1981). https://doi.org/10.1016/0022-2011(81)90132-4.

41. Bøhn, T., Rover, C., Semenchuk, P. *Daphnia magna* negatively affected by chronic exposure to purified Cry-toxins. *Food and Chemical Toxicology.* **91**. 10.1016/j.fct.2016.03.009. (2016).

42. Chen, Y. et al. Safety of *Bacillus thuringiensis* Cry1C protein for *Daphnia magna* based on different functional traits. *Ecotoxicology and environmental safety*, **147**, 631–636. (2018). https://doi.org/10.1016/j.ecoenv.2017.08.065.

43. Dieter, E. *Daphnia* as a versatile model system in ecology and evolution. EvoDevo. **13**. 10.1186/s13227-022-00199-0. (2022).

44. Lampert, W. Studies on the carbon balance of *Daphnia pulex* de Geer as related to environmental conditions. Determination of the threshold concentration as a factor controlling the abundance of zooplankton species. *Archiv Fur Hydrobiologie,* **4834**: 361-368. (1977).

45. Glare, T. and O'Callaghan, M. *Bacillus thuringiensis*: Biology, Ecology and Safety. Chichester; Wiley, 2000.

46. Schiwy, S., Braunigg, J, Alert, H., Hollert, H., Eiter, S. A novel contact assay for testing aryl hydrocarbon receptor (AhR)-mediated toxicity of chemicals and whole sediments in zebrafish (*Danio rerio*) embryos. *Environmental Science and Pollution Research*, **22**, 16305, 1. (2015).

47. Nigro, L. et al. Unveiling the multilevel impact of four water-soluble polymers on *Daphnia magna*: from proteome to behaviour (a case study), *Journal of Hazardous Materials*, **469**,134000. (2024) https://doi.org/10.1016/j.jhazmat.2024.134000.

48. Leuthold, D., Iver, N., Altenburger, R., Busch, W. Can environmentally relevant neuroactive chemicals specifically be detected with the locomotor response test in zebrafish embryos? *Environmental Scientific Technologies,* **53**, 82–93. (2019).

49. Bradford, M., M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry,* **72**, 248–254. (1976). https://doi.org/10.1016/0003-2697(76)90527-3

50. Della Torre, C. et al. The interactions of fullerene C60 and Benzo(α)pyrene influence their bioavailability and toxicity to zebrafish embryos. *Environmental pollution* (Barking, Essex: 1987), **241**, 999–1008. (2018). https://doi.org/10.1016/j.envpol.2018.06.042

51. Ellman, G. L., Courtney, K. D., Andres, V., Jr, Feather-stone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, **7**, 88–95. (1961). https://doi.org/10.1016/0006-2952(61)90145-9

Fig. 1. Swimming behavior of zebrafish larvae following a 120-hour exposure to various MosChito raft compositions. **(A)** Total distance moved measured as total mm and **(B)** mobility measured as mean (%). Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeasts (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Values represent the mean ± standard error (SE) of three independent biological replicates.

Fig. 2. Biomarkers in *D. rerio* zebrafish larvae after exposure to MosChito rafts. (A) Reactive oxygen species (ROS) levels, expressed as fluorescence arbitrary units (FAU) per mg of protein, and **(B)** acetylcholinesterase (AChE) activity, measured as nmol/min/mg of protein, are shown for each treatment group: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeasts (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Values represent the mean ± standard error (SE) of three independent biological experiments.

Fig. 3. Swimming behavior of *D. magna* following a 15-day exposure to MosChito rafts. **(A)** Total distance moved, measured as total mm. **(B)** Mobility, measured as average (%) of time spent in active movement. Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeast (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Data are presented as mean ± standard error (SE) of three independent biological experiments; different letters indicate statistically significant differences (**Supplementary Table 1**).

Fig. 4. Analysis of biomarkers in *D. magna* after exposure to MosChito rafts. (A) Reactive oxygen species (ROS) levels, expressed as fluorescence arbitrary units (FAU mg^{-1}protein). **(B)** Acetylcholinesterase (AChE) activity, expressed as nmol $min^{-1}mg^{-1}$ protein. Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeast (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). Data are presented as mean ± SE for each group of three independent biological experiments; different letters above the bars indicating statistically significant differences between groups.

Fig. 5. Impact of MosChito rafts on the reproductive capacity of *D. magna* over 14 days. (A) Daily mean of neonates across the 14-day period. **(B)** Average number of neonates produced per treatment group over the entire 14-day period. Treatment groups: control (Ctrl); empty hydrogel (Matrix); hydrogel with yeast (Yeast); hydrogel with VectoBac 12AS (*Bti*); hydrogel with yeasts and VectoBac 12AS (*Bti*+Yeast). The Data are presented as mean ± SE of three independent experiments.

Author contributions statement

Negri Agata: Methodology, Investigation, Formal analysis, Writing - Original Draft, Project administration **Nigro Lara**: Methodology, Investigation, Software, Writing - Review & Editing **Signorini Silvia Giorgia**: Methodology, Investigation, Formal analysis **Locarno Silvia**: Methodology, Resources **Diana Alberto**: Methodology, Resources **Caccia Silvia**: Writing - Review & Editing, Validation **Gabrieli Paolo**: Data Curation, Validation, Writing - Review & Editing **Binelli Andrea**: Resurces, Validation, Visualization **Del Giacco Luca**: Resurces, Validation, Visualization **Bandi Claudio**: Supervision, Funding acquisition **Della Torre Camilla**: Supervision, Visualization, Investigation, Data Curation **Epis Sara**: Conceptualization, Supervision , Project administration