

# The combination of unicellular green algae from the Scenedesmaceae family (Chlorophyta) and inorganic fertiliser favors the proliferation of the mosquito *Aedes albopictus* (Diptera: Culicidae)



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## Abstract

Inorganic fertilisers (IF) are widely used often leading to pollution of breeding sites of mosquitoes. In the same locations many species of unicellular algae, on which mosquito larvae feed, can be found as well. In this experiment, three conditions were studied: water, IF alone and an IF solution in which green algae belonging to the Scenedesmaceae family were cultivated (IF + A). When *Aedes albopictus* female mosquitoes could not choose where to lay their eggs because they had no possibility of contact with the different treatments beforehand – in the bioassays performed in blind double-tunnels – the numbers of positive or negative breeding sites and mean egg number in each were not statistically different. In contrast, when the females could freely choose where to lay their eggs – in the bioassays performed in plastic containers – the largest number of eggs were found in the IF + A treatment, followed by IF alone and, finally, water alone. The specific attractiveness of IF was clearly correlated with the greater carbon dioxide emissions of this treatment compared with the other two. This observation thus revealed that the carbon dioxide released by the algae accounts for the selection of breeding sites. Besides being more attractive to *A. albopictus* females, the IF + A combination ensured greater survival rates to the larvae and pupae.

**Key words:** *Aedes albopictus*, blind double-tunnel, carbon dioxide, inorganic fertiliser, Scenedesmaceae algae family.

**Abbreviations:** A, algae; IF, inorganic fertiliser.

## Introduction

Survival or death of generations of mosquitoes depend on oviposition behaviour (Subra 1971). Originating from South-East Asian tropical forests, *Aedes albopictus* (Skuse 1894) (Diptera: Culicidae) is a mosquito of great genetic plasticity, which has allowed it to adapt to a large variety of environments. The gravid females of *A. albopictus* lay their eggs one by one in small to medium-sized containers such as jars, drinking water storage canisters, plant saucers, cans, tires, troughs along with dozens of natural tiny breeding sites scattered in green areas and yards (Cordellier et al. 1977). The choice of a breeding site will depend on many physicochemical and biological factors, the most significant being the volume of the container and the biomass present in the water (Barrera et al. 2006; Darriet, Corbel 2008; Darriet et al. 2010), the density of larvae and pupae of the same species (Soman, Reuben 1970; Roberts, His 1977; Bentley, Day 1989; Darriet, Corbel 2008) and concentration of nitrogen (N), phosphorus (P)

and potassium (K) (Darriet, Corbel 2008; Darriet et al. 2010). The gravid female mosquitoes looking for a breeding site are attracted by water where inorganic fertiliser (IF) is present, the mineral salts favouring the development of supplementary biomass made of algae, bacteria and fungi (Hazard et al. 1967; Trexler et al. 2003; Hao et al. 2010). This supplementary food biomass in breeding sites is crucial for the mosquito larvae because they cannot digest cellulose (Clement 2000). There is evidence that water containing organic matter and inorganic fertiliser increases the mosquito survival rates two to three-fold compared with water containing no fertiliser (Darriet 2018).

The experiments carried out in the scope of this study used green unicellular algae from the Scenedesmaceae family (order: Sphaeropleales). Depending on the species the green micro-algae can be either round, oblong or fusiform. They also gather in cenobes (clusters of several cells) (Hegewald, Hanagata 2000). The impact of inorganic fertiliser + algae combination (IF + A) on the oviposition behaviour of mosquitoes was determined in comparison

with deionised water (water) and IF alone using blind double-tunnels in which the female mosquitoes were not able to touch the water of the breeding sites prior to laying. Another experiment was carried out with standard plastic containers where contact with water was possible. The adult emergence rates of *A. albopictus* were recorded in all three treatments as well as the carbon dioxide (CO<sub>2</sub>) quantities released by each of them over a period of ten days.

## Materials and methods

### Inorganic fertiliser

The 7-5-6 NPK composition of inorganic fertiliser (universal fertiliser, liquid formulation, Algoflash®, Compo France SAS, Roche-Lez-Beaupré) was 7% total nitrogen (N) with 3.6% ureic nitrogen, 1.3% ammonium nitrogen (NH<sub>4</sub><sup>+</sup>), and 2.1% nitric nitrogen (NO<sub>3</sub><sup>-</sup>); 5% phosphorous (P) in the form of phosphoric anhydride (P<sub>2</sub>O<sub>5</sub>) and 6% potassium (K) in the form of potassium oxide (K<sub>2</sub>O); 1% sulfur trioxide (SO<sub>3</sub>) and 0.1% sodium oxide (Na<sub>2</sub>O). The trace elements present in the formulation were 0.01% boron (B); 0.02% copper (Cu); 0.02% iron (Fe); 0.01% manganese (Mn); 0.001% molybdenum (Mo) and 0.002% zinc (Zn).

### Biological material

The unicellular green algae (A) used in this study belong to the Scenedesmeaceae family (order Sphaeropleales). The specific strain was collected from under a flower pot located in the village of Perols (Hérault department). The identification of the microalgae under microscope was performed at the Centre d'Etude et de Valorisation des Algues (CEVA), Pleubian – France.

All bioassays were conducted with either stage 1 larvae or gravid females of tiger mosquito from the *Aedes albopictus* Perols strain.

### The bioassays

The biological tests carried out in this study were performed at temperature of 27 ± 2 °C and a relative humidity of 70%. The bioassay lighting system was run using a 14-h-day (6:00 to 20:00) and 10-h-night programme (20:00 to 6:00). The light source used was a LED light bulb, Philips A60 E27, 7 W, providing nominal luminous flux of 806 lm.

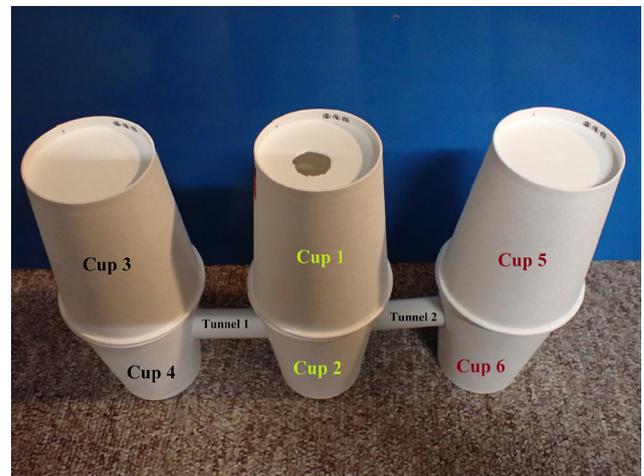
### Test composition

Medium consisting of deionised water only was used as a control. The concentrations of NPK used in the IF treatment were 17 mg L<sup>-1</sup> of nitrogen, 12 mg L<sup>-1</sup> of phosphorous and 14 mg L<sup>-1</sup> of potassium. These NPK concentrations were calculated from the 7-5-6 NPK composition of inorganic fertiliser. The medium used for inorganic fertiliser + microalgae (IF + A) contained the same concentration of fertiliser as that used in the IF treatment, to which the fertiliser was added once a week. After a two-week period

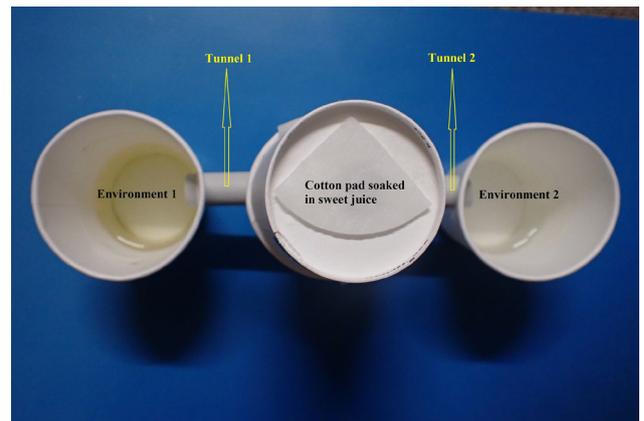
of culture at a 27 ± 2 °C temperature, IF + A cultures were used to carry out the bioassays. The exact amounts of algae in the IF + A treatments could not be determined due to lack of appropriate equipment.

### Bioassays in blind double-tunnels

This evaluation technique specifically elaborated for this study consisted in placing a female of *A. albopictus* inside an experimental device preventing any contact with the treatments before laying (Fig. 1). One freshly blood-fed *A. albopictus* female was introduced in the sluice made with cups 1 and 2. Once the female was isolated, a cotton pad soaked in sweet juice (water + sugar 1%) was placed at the same entry by which the female was introduced. Cups 3 & 4 and 5 & 6 contained the treatments to be tested. Cups 4 and 6 were lined with a filter paper strip. Each of these two cups contained 50 mL of water, IF or IF+A combination (Fig. 2).



**Fig. 1.** Three-quarter view of a blind double-tunnel. The effects of inorganic fertiliser + algae (IF + A) on the oviposition behavior of the mosquito were determined in comparison with deionised water and inorganic fertiliser alone (IF) (without any direct contact of the female mosquito with the treatments) (© Frédéric Darriet).

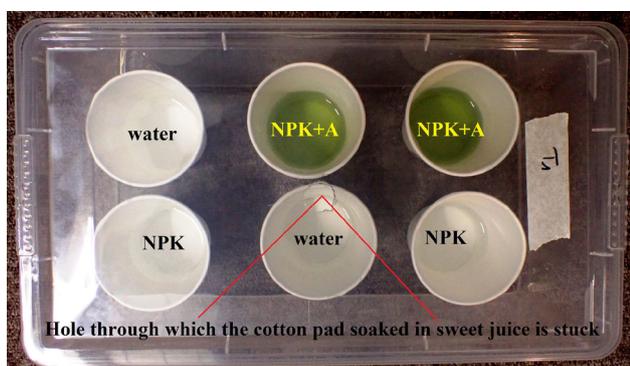


**Fig. 2.** Top view of a blind double-tunnel. The photograph shows the inside of both laying chambers with the treatments to be tested (© Frédéric Darriet).

The pairs tested were as follows: pair 1, water versus IF; pair 2, water versus IF + A; pair 3, IF versus IF + A, each of them being tested on 45, 42 and 47 replicates, respectively. The female introduced in the sluice (cups 1 and 2) could choose the most attractive treatment as the sluice was connected to both breeding sites via two opposite tunnels. Seventy two hours later, the numbers of eggs laid on the filter paper strips were counted.

#### Bioassays in plastic containers

Using bioassays in plastic containers, blood-fed *A. albopictus* females were in contact with the breeding sites prior to laying. The plastic containers allowed a simultaneous evaluation of the experimental conditions over a 72-h period. The position of the water control, IF and IF + A cups inside the containers was randomly selected for each replicate (Fig. 3). The containers were 0.33 m long, 0.19 m wide and 0.12 m high boxes made of plastic. All three treatments were distributed among six cups (two cups per treatment). As with the blind double-tunnels experiments, all cups were lined with a white fiber paper strip inside. Fifty mL of water, IF or IF + A were then added. Ten blood-fed females were introduced in each plastic container. Six



**Fig. 3.** The plastic containers allow the females of *Aedes albopictus* to have direct contact with the treatments before laying (© Frédéric Darriet).



**Fig. 4.** Experimental device used to measure the carbon dioxide released by the water, inorganic fertiliser alone (IF) and inorganic fertiliser + algae (IF + A) treatments. Temperature (in °C) and hygrometry (in % relative humidity) were recorded at 9:00 and 21:00 (© Frédéric Darriet).

replicates of 10 females were performed with a random rotation of the treatments each time. Seventy two hours later, during which sweet juice (water + sugar 1%) was given to the females to maintain survival, the eggs laid on the filter paper strip were counted.

#### Mortality rates of *A. albopictus* preimaginal stages

Larval bioassays were carried out using water, IF and IF + A solutions. Groups of 50 stage 1 larvae were placed in 100 mL of aliquot in 200 mL paper cups. Ten cups ( $n = 500$  larvae) were used for each treatment. No food was supplied to the larvae in the cups, the nutrients coming only from the constitutive elements of each treatment. The larvae and pupae deaths were counted in each cup to determine the mortality rates in each treatment. Mortality was determined until the last imaginal emergence or larval death, ranging from one to two months.

#### Estimation of carbon dioxide released

The CO<sub>2</sub> concentration [expressed in parts per million (ppm)] was measured with a carbon dioxide detector (PG-L28A-CO<sub>2</sub> model; CO<sub>2</sub> detection range: 400 to 5000 ppm; CO<sub>2</sub> detection sensitivity: 1 ppm) every 24 h during 10 days. The same device allowed the recording of both temperature (in °C) and hygrometry (in % relative humidity) at the time of reading. Measures were made at 9:00 and 12 h later, at 21:00. The experimental device consisted in three 0.33 m long, 0.19 m wide and 0.12 m high plastic containers in which 1 L of water, IF or IF + A was introduced. The carbon dioxide detector was then put inside a small plastic box to keep it away from water.

#### Statistical analysis

The statistical comparisons were performed with Statistica V10 software (Statistica 2011). The data collected were subject to non-parametric analyses (non-Gaussian distribution). For bioassays carried out in blind double-tunnels the statistical analysis was performed using the Kolmogorov-Smirnov test comparing the number of eggs laid in the pairs (water versus IF), (water versus IF + A) and (IF versus IF + A) treatments. For bioassays performed in plastic containers with all three treatments together, the statistical comparison was made using a Kruskal-Wallis test. The same statistical analysis method was also used to compare the mortality rates of the larvae and pupae of *Ae. albopictus* in the three treatments studied. All carbon dioxide (CO<sub>2</sub>) measures were analysed using the Kruskal-Wallis test. Statistical comparisons were considered significant when  $p < 0.05$ .

## Results

#### Bioassays in blind double-tunnels

For all pairs tested (water versus IF, water versus IF + A, and IF versus IF + A), the numbers of positive or negative

**Table 1.** Number of the *Aedes albopictus* egg-negative or positive breeding sites recorded in the blind double-tunnels. The tests were carried out with water versus inorganic fertilizer (IF), water versus inorganic fertilizer + algae (IF + A) and inorganic fertilizer (IF) versus inorganic fertilizer + algae (IF+A) pairs. <sup>abc</sup>, the bioassays were respectively performed on 45<sup>a</sup>, 42<sup>b</sup> and 47<sup>c</sup> replicas of blind double-tunnels. <sup>d</sup>, the statistical analysis was performed using chi-deux (table 2 x 2 ; *dl* = 1) (non-Gaussian distribution).

Treatment	Water <sup>a</sup>	IF <sup>a</sup>	Total	<i>P</i> <sup>d</sup>	Water <sup>b</sup>	IF + A <sup>b</sup>	Total	<i>P</i> <sup>d</sup>	IF <sup>c</sup>	IF + A <sup>c</sup>	Total	<i>P</i> <sup>d</sup>
Negative breeding sites	22	22	44	1.0	19	18	37	0.83	19	21	40	0.68
Positive breeding sites	23	23	46		23	24	47		28	26	54	

sites with *A. albopictus* eggs laid were not statistically different ( $1.0 < p > 0.68$ ) (Table 1). Also the mean number of eggs laid was not significantly different between the three treatments ( $p > 0.1$ ) (Table 2).

**Bioassays in plastic containers**

The IF treatment doubled the attractiveness of the site compared with water ( $p = 0.029$ ). The IF + A treatment also proved to be twice as attractive as IF ( $p = 0.0044$ ) and five times that of water ( $p = 0.0000001$ ) (Fig. 5).

**Mortality rates of *A. albopictus***

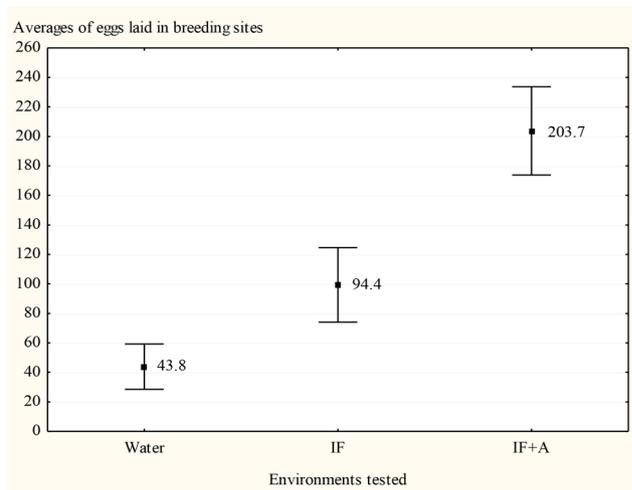
The statistical analysis did not evidence significant differences in the mortality rates in water and IF treatments ( $p = 1$ ). However, the differences were statistically significant between water and IF+A ( $p = 0.00034$ ) and between IF and IF+A ( $p = 0.00063$ ) (Fig. 6).

**Amount of carbon dioxide (CO<sub>2</sub>) released**

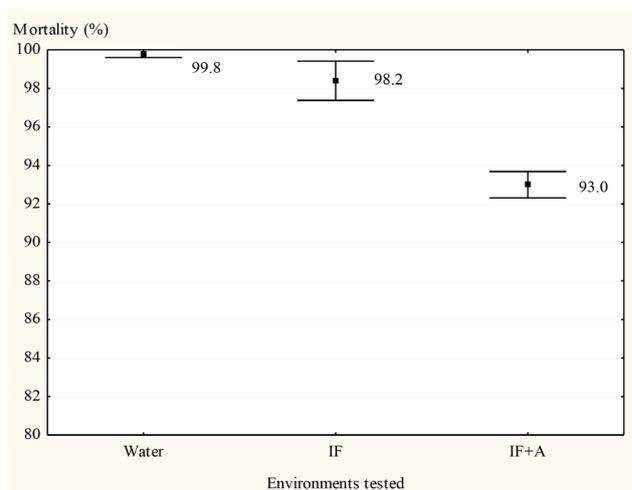
During the 10 days the carbon dioxide amounts were recorded, the average temperature was 22.2 °C (16 to 28 °C) with relative hygrometry of 79.4% (65 to 83%). According to the reading done at 9:00, the CO<sub>2</sub> concentrations were significantly different between IF + A versus water and IF + A versus IF ( $p = 0.000007$  and  $p = 0.014$ ; respectively) yet there was no significant difference between water versus IF ( $p = 0.17$ ) (Fig. 7A). The statistical analysis of the measures

**Table 2.** Averages of eggs laid by the *Aedes albopictus* females in the breeding sites containing water, inorganic fertilizer (IF) and inorganic fertilizer + algae (IF + A) solutions. The tests were carried out on water versus IF, water versus IF + A and IF versus IF + A pairs in blind double-tunnels. <sup>abc</sup>, the bioassays were respectively performed on 45<sup>a</sup>, 42<sup>b</sup> and 47<sup>c</sup> replicas of blind double-tunnels. <sup>d</sup>, the statistical analysis was performed using the Kolmogorov-Smirnov test (non-Gaussian distribution)

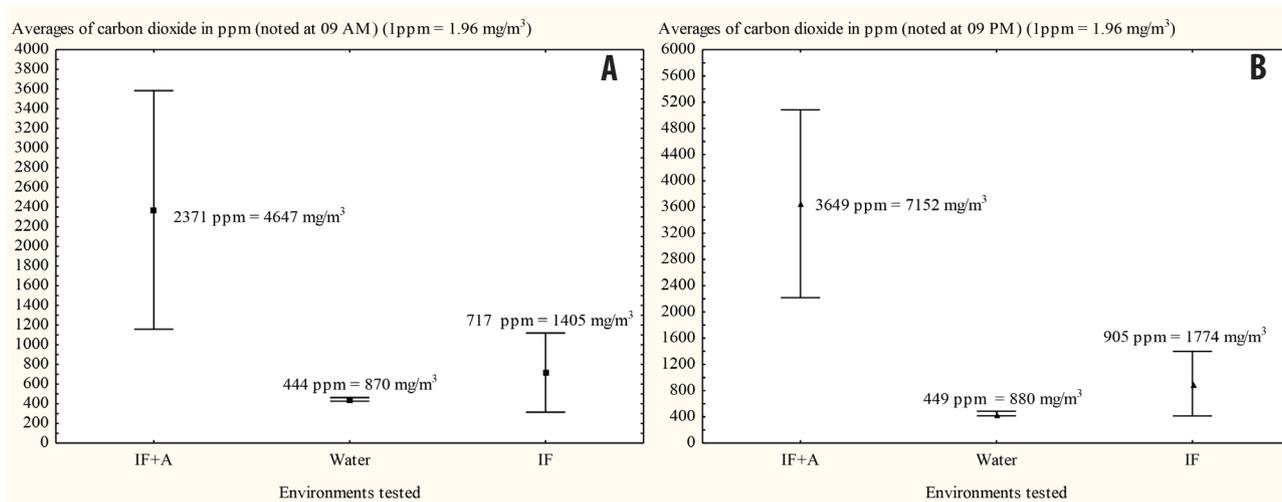
Treatment	Eggs averages (IC95 %)	<i>P</i> <sup>d</sup>
Water <sup>a</sup>	69.1 (54.1 to 84.1)	> 0.1
IF <sup>a</sup>	60.7 (49.4 to 72.0)	
Water <sup>b</sup>	53.8 (42.8 to 64.8)	
IF + A <sup>b</sup>	57.6 (47.5 to 67.7)	
IF <sup>c</sup>	60.8 (50.1 to 71.5)	
IF + A <sup>c</sup>	70.7 (59.8 to 81.6)	



**Fig. 5.** Means number of eggs laid (experiment carried out in plastic containers) by *Aedes albopictus* females in water, inorganic fertiliser alone (IF) and inorganic fertiliser + algae (IF + A) treatments. The means and confidence intervals (CI 95%) were calculated on a total of 18 replicas for each treatment. The statistical analysis was performed using the Kruskal Wallis test (non-Gaussian distribution).



**Fig. 6.** Mortality rates (%) (CI 95%) of larvae and nymphs of *Aedes albopictus* recorded in the water, inorganic fertiliser alone (IF) and inorganic fertiliser + algae (IF + A) treatments. The statistical analysis was performed using the Kruskal Wallis test (non-Gaussian distribution).



**Fig. 7.** The mean carbon dioxide (CI 95%) concentrations recorded at 9:00 (A) and 21:00 (B) in plastic boxes containing water, inorganic fertiliser alone (IF) or inorganic fertiliser + algae (IF + A) treatments. The statistical analysis was performed using the Kruskal Wallis test (non-Gaussian distribution). The mean carbon dioxide concentrations are expressed in ppm and mg m<sup>-3</sup>, knowing that 1 ppm of CO<sub>2</sub> is equivalent to 1.96 mg m<sup>-3</sup>.

recorded at 21:00 revealed significant differences between all three treatments ( $0.033 < p > 0.000001$ ) (Fig. 7B).

## Discussion

Even if mosquitoes lay their eggs anywhere in a natural or anthropised environments, there is evidence that factors related to the quality of the water will make some sites more attractive than others. Decaying leaves or hay have long been known for their attractiveness for mosquitoes (Scott et al. 1967; Chadee et al. 2001). Proliferating algae and bacteria also have a powerful attractiveness (Hazard et al. 1967; Trexler et al. 2003; Kaufman et al. 2006). There is evidence that breeding sites containing fertilisers are favoured by female mosquitoes (Darriet, Corbel 2008; Darriet et al. 2010; Anderson, Davis 2014; Darriet 2018; Ahmad-Azri et al. 2019). If the presence of biomass, whether dead or alive, and fertilisers in breeding sites is already known for attracting female mosquitoes, it is still not known which factors contribute most to such attractiveness.

The experiments carried out within the frame of this study have allowed to better grasp how *A. albopictus* behaves when it comes to choosing a breeding site, whether there is just water or water and inorganic fertiliser (IF) or water containing both fertilisers and unicellular green algae from the Scenedesmeaceae family (Chlorophyta division of plants) (IF + A). When the *A. albopictus* females had no direct contact with the waters of breeding site before laying their eggs, the numbers of egg-negative or positive breeding sites and the mean egg number laid in each did not show any statistically significant differences. However, when the females had contact beforehand, the number of eggs laid were greater in IF + A ( $\times 4.7$  compared with water alone;  $\times 2.2$  compared with IF) and the IF treatment attractiveness

was 2.2 times higher than the water treatment. This study confirms the attractiveness of inorganic fertilisers to gravid mosquito females. Also, water containing algae along with fertilisers (IF + A) was significantly more attractive.

As all aerobic organisms, green algae breathe by consuming dioxygen (O<sub>2</sub>) and releasing carbon dioxide (CO<sub>2</sub>) which irresistibly attracts female mosquitoes (Guerenstein, Hildebrand 2008; Burkett-Cadena et al. 2015; Ellwanger et al. 2021; Barredo et al. 2022). This study also revealed that carbon dioxide plays a part in the choice of breeding sites. Indeed the CO<sub>2</sub> concentration in IF + A treatments reached five and eight times greater peaks at 9:00 and 21:00, respectively, than the amount recorded with water. The carbon dioxide accumulation in the IF + A treatments suggests that the cellular respiration of the algae (CO<sub>2</sub> release) always prevailed over the chlorophyll-dependent process (CO<sub>2</sub> absorption). The gas imbalance was most likely correlated to the particular light conditions during the bioassays. Therefore, further experimentation in order to get a better understanding of the CO<sub>2</sub> release and absorption balance is needed, exploring different light intensities including absolute darkness.

Fertiliser is not directly assimilated by mosquito larvae. However, the three minerals (nitrogen, phosphorus and potassium) promote the development of bacteria, algae and fungi, increasing the food biomass of the breeding sites. Mosquito larvae exploit this additional biomass to proliferate, which incidentally accounts for lower larval and pupal mortalities in the IF + A treatment (93%) compared with water and IF (99.8 and 98.2%, respectively). In light of all these observations there is no question that the sometimes injudicious use of fertilisers in agriculture generates new ecological situations favouring mosquito proliferation. Interestingly, removing algal mats from

larval habitats proves a promising control measure for mosquito populations (Bond et al. 2004). In contrast, it would be just as promising an approach to take advantage of the attractiveness of inorganic fertiliser and unicellular green algae to devise innovative control strategies against mosquitoes, vectors of pathogens to man. These findings could, for example, lead to the development of new traps that would attract female mosquitoes and kill hatched larvae if mixed with appropriate larvicides.

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