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REVEALING MICROBIAL COMPONENTS IN BIOFILM ON AQUATIC INSECT CADAVERS: AN EXPERIMENTAL TAPHONOMIC STUDY

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ABSTRACT – The La Cantera Formation, recognized as a *Konservat-Lagerstätten*, preserves exceptional aquatic insect fossils from the Early Cretaceous. This study explores, using experimental taphonomy, the role of microbial biofilms in the preservational process over aquatic insects. An experimental analysis is proposed to understand the impact of microorganisms on decomposition and preservation. The investigation focuses on aquatic insects as model organisms because they are relevant in the La Cantera insect record. The experiment, conducted in controlled conditions, includes inhibitors targeting algae, bacteria, and fungi, revealing the development of biofilms and their impact on insect preservation. The differential development of microorganisms under each treatment indicates that biofilms, composed of algae and fungi, significantly impact the preservation of corpses by preventing disarticulation and facilitating preservation. This suggests that microbial mats could be crucial in forming exceptional paleontological deposits. By comparing these findings with the fossil record from La Cantera, researchers may gain insight into the potential significance of microbial mats in forming such deposits. This research highlights the importance of understanding the role of microorganisms in preserving fossils and contributes to our understanding of paleontological processes.

Keywords: experimental taphonomy, Heteroptera, microbial activity, La Cantera Formation.

RESUMO – A Formação La Cantera, reconhecida como um *Konservat-Lagerstätten*, preserva fósseis excepcionais de insetos aquáticos do Cretáceo Inferior. Este estudo explora o papel dos biofilmes microbianos no processo de preservação desses insetos. Uma análise experimental foi proposta para compreender o impacto dos microrganismos na decomposição e na preservação. A investigação é focada nos insetos aquáticos como organismos modelos porque são relevantes no registro de insetos de La Cantera. O experimento, conduzido em condições controladas, inclui inibidores direcionados às algas, às bactérias e aos fungos, revelando o desenvolvimento de biofilmes e seu impacto na preservação dos insetos. O desenvolvimento diferencial de microrganismos em cada tratamento indica que os biofilmes, compostos por algas e fungos, impactam significativamente a preservação dos cadáveres, evitando a desarticulação e facilitando a preservação. Isto sugere que os tapetes microbianos podem ser cruciais na formação de depósitos paleontológicos excepcionais. Ao comparar estas descobertas com o registro fóssil de La Cantera, os investigadores podem obter informações sobre a importância potencial dos tapetes microbianos na formação de tais depósitos. Esta pesquisa destaca a importância de compreender o papel dos microrganismos na preservação de fósseis e contribui para a nossa compreensão dos processos paleontológicos.

Palavras-chave: tafonomia experimental, Heteroptera, atividade microbiana, Formação La Cantera.

INTRODUCTION

Some authors considered the La Cantera Formation as *Konservat-Lagerstätten* in the strict sense of the term (Seilacher, 1990; Castillo Elías, 2016). This unit is a fluvial-lacustrine environment in which the physicochemical conditions during sedimentation have been unique enough to preserve delicate organisms and, in some cases, their soft tissues. According to Petrulevicius *et al*. (2010), the relatively exceptional insect preservation could be linked to bacterial sealing (produced by some biofilm or microbial mat) that would have acted on the sediment surface, protecting organisms from natural degradation. The studied fossil insects were preserved in mudrocks deposited in a shallow paleolake

environment that indicates quiet water (Arcucci *et al*., 2015; Castillo Elías, 2016). The La Cantera Formation displays evidence of wrinkles in the association of lacustrine facies. In the shoreface facies association, wrinkles, syneresis cracks, and kidney structures are evidence of the former presence of microbial mats (Castillo Elías, 2016). However, no direct evidence on top of the fossil insects was documented.

Some authors (Seilacher, 1985; Briggs, 2003; Kaye *et al*., 2008; Wang *et al*., 2012; Raff *et al*., 2014) have proposed that microbial communities play a pivotal role in the selective preservation of organic remains. This preservation is attributed to various factors, including reduced available oxygen, nutrient competition, pH alteration, physical barrier creation, and tissue preservation. Consequently, these microbial communities form a protective layer surrounding the remains, shielding them from erosion and degradation and facilitating ion concentrations conducive to soft tissue mineralization. Furthermore, the formation of a microbial "sarcophagus" surrounding the insect corpse produces a harmful mold within its inner layer, faithfully preserving the original morphology of the insect with remarkable fidelity. Once this microbial "sarcophagus" is established, previous observations indicate a gradual degradation compared to other corpses lacking the formation of microbial mats (Iniesto *et al*., 2016, 2020; Tian *et al*., 2020). The physical and chemical conditions produced after the isolation of the body favor preservation through different mechanisms, like organic preservation, mineralization, and the formation of molds (Iniesto *et al*., 2016, 2020; Tian *et al*., 2020).

While countless insect species thrive in contemporary ecosystems and the fossil record, fossilized insects remain relatively scarce. The preservation of these fossils involves various mechanisms, each leading to different levels of preservation fidelity. Notably, during the Cretaceous Period, a rich paleoentomofauna has been documented in several successions, such as Las Hoyas (Spain) and Crato Formation (Brazil). Specifically, these successions consist of limestone traditionally attributed to chemical precipitation and fine terrigenous sediments influenced by microbial organic mediation (Martínez-Delclòs *et al*., 2004; Bezerra *et al*., 2020; Dias & Carvalho, 2020; Iniesto *et al*., 2020).

The La Cantera Formation records the presence of insect fossils (Figure 1) from orders such as Coleoptera, Heteroptera, Hymenoptera, and Dermaptera, with the majority of the fossil remains belonging to the order Heteroptera (Sallenave, 2003; Petrulevicius *et al*., 2010; Janello, 2012; Arcucci *et al*., 2015; Castillo Elías, 2016; Sierra, 2019). The aquatic insects recorded were assigned to three families: Notonectidae, Corixidae, and Belastomatidae (Sallenave, 2003; Petrulevicius *et al*., 2010; Janello, 2012). Besides the record of *Notonecta mazzoniae* and the presence of the older representative of the Subfamily Anisopinae (Petrulevicius *et al*., 2010), it was also confirmed the presence of exuvius of Notonectidae (Janello, 2012).

The exceptional preservation of aquatic insects and their exuviae in the La Cantera Formation raises questions about the fossilization process and the potential involvement of microbial mats. The absence of direct evidence of microbial mat over La Cantera aquatic insects triggers an experimental taphonomy analysis that proposed to assess the impact of various microorganisms on the decomposition and preservation of aquatic insect corpses. This study aimed to ascertain the timing of biofilm formation and its components on the bodies of aquatic insects from small streams near San Luis City in mountainous environments. This investigation seeks to compare the preservation patterns observed in fossil remains of similar insects from the La Cantera Formation.

MATERIAL AND METHODS

For the taphonomic experiment, samples of aquatic heteropteran insects were collected from the banks of the El Volcán River (Figure 2), which is part of the Bebedero River Basin, SW of the Sierras de San Luis, biogeographic province of Chaco, Serrano District (Ojeda *et al*., 2016). The El Volcán River has an irregular pluvial regime that is

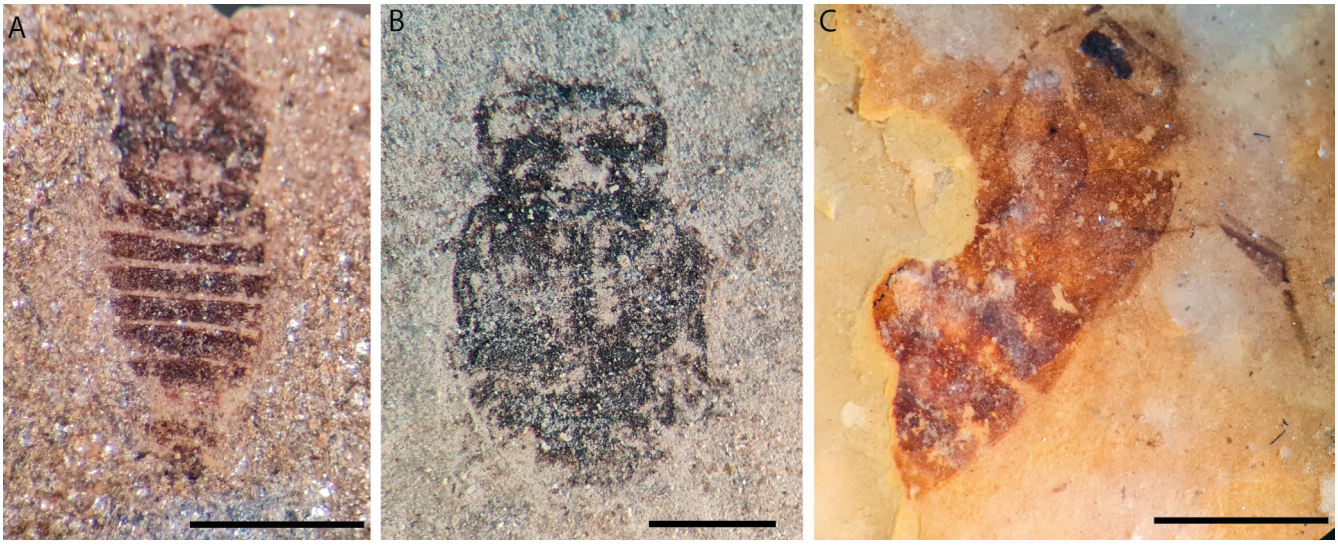

Figure 1. Insects from La Cantera Formation. Scale bars = 1 mm.

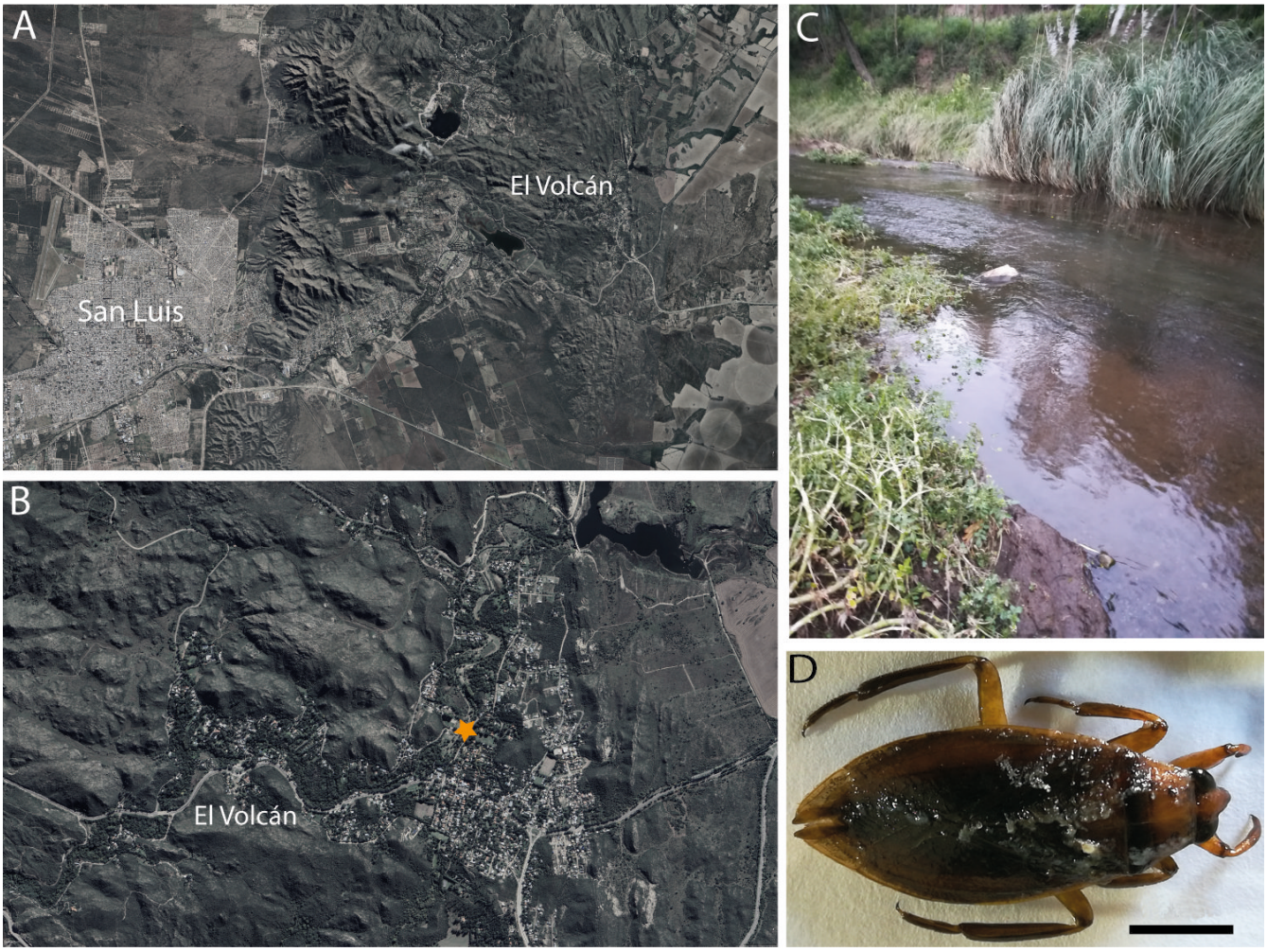

Figure 2. A. Satellite image with location of sample site El Volcán River, El Volcán locality, Juan Martin de Pueyrredón Department, San Luis, Argentina. (33°14'53.11"S 66°10'45.86"W). **B**. Photo of the sample site. **C**. *Belostoma bifoveolatum* was selected as the model organism for the experiments. Scale bar = 10 mm.

permanent during the year. In winter, water comes mainly from underground sources, while in summer, the flux increases from the rain. The soil in the area is deep and fertile, with some hydric erosion. The model organism selected for the experiments is *Belostoma bifoveolatum* (Figure 2), Suborder Heteroptera (Coscarón, 2017). The model organism was selected for two main reasons: its abundance and favorable size for conducting observations during the experiment, and it has a close taxonomic and phylogenetic relationship with the fossil insects from the La Cantera Formation. The insects were collected from river sectors with aquatic vegetation and without current. They were collected with water hand nets, taken to sterile jars, and labeled. In the laboratory, the insects were frozen to avoid decomposition until the start of the experiment.

The water samples were taken from the same riverbanks on the shallow riversides, with abundant vegetation, rocks, and sand bottoms. Two samples were taken in the different fluvial conditions of the river in glass jars previously sterilized. Other samples were taken from different river sectors in plastic containers and were used to mount the experiment. A culture medium (PCA-agar for plaque counting) was prepared to have the necessary nutrients to grow microorganisms from the collected water. In addition, a buffer saline phosphate pH 7.4 was ready to make serial dilutions that allowed posterior isolation from the microorganisms of the samples.

A preliminary taphonomic experiment was conducted to refine and optimize the design of the definitive experiment. In this initial study, six treatments were implemented (Table S1), with the primary objective of promoting the growth of specific microorganisms while inhibiting others. The insights gained from the preliminary experiment informed the selection of the five treatments with varying combinations of chemicals (Table 1) for the definitive experiment. These treatments were

Table 1. Components in each treatment. Algaecide Nataclor 10 µL. Antibiotic Estreptomicina 0.012 grs. Fungicide Carbendazim: 50 µL.

Treatment	Components
T1	CONTROL (*only water of river)
T2	ALGAECIDE + ANTIBIOTIC
T3	ALGAECIDE + FUNGICIDE
T4	ANTIBIOTIC + FUNGICIDE
T5	ANTIBIOTIC + FUNGICIDE + ALGAECIDE

carefully chosen to facilitate a more accurate inference and interpretation of the processes and components influencing biofilm formation. It was carried out in the summer between January and March 2019. Five treatments were set, with six replicas on each one, forming a total set of 30 experimental units. Labeled and sterilized Petri dishes were used to mount the experiment, as shown in Supplementary Table 2. Each Petri dish was set on the laboratory table and randomly disposed of in three lines of 10 dishes.

Each one contained 200 ml of river water with a mark indicating the liquid level to maintain the same volume during the experiment in case of evaporation, with the addition of distilled water. A different combined chemical was added to each treatment. Thus, 200 ml of water was added to different inhibitors: 10 µl algaecide Natacloror, or 0.012 gr antibiotic Estreptomicina, or 50 µl of fungicide Carbendazim. Four corpses of insects were added to each dish. The weight of each insect was measured with an analytic scale. Each dish had insects with equivalent weight and size in each experimental unit.

The conditions in the laboratory were a photoperiod of 12 hours of light and 12 hours of darkness. A mercury thermometer measured the air temperature daily during the 35 days the experiment was conducted at the same hour. The water characteristics, like the pH and the temperature, were also measured in each experimental unit. The pH was measured with the plastic pH test strips. The universal pH strips contain a combination of indicators that cover a range of pH values up to and including 0 to 14 in a single test, pH 0-14 color chart for quickly comparing the test results with the four colors pH panels on the cardboard, to obtain the solution pH. The water temperature was measured with an alcohol thermometer.

All the values were recorded along the 35 experimental days, on days 1, 2, 5, 7, 9, 12, 15, 20, 25, 30, and 35. The pH and water temperature measurements were made simultaneously with the insect sampling in each dish on the same days. The insect sampling to observe the corpses in detail was taken randomly from five dishes (one corresponding to each treatment) and one corpse of an insect to observe under a stereoscopic binocular microscope (Olympus brand). In the meantime, the observation considered not only the qualitative characteristics of the insect body, like its position, color changes, the occurrence of stains, breaks in the cuticle, or disarticulation, but also the water-color changes and turbidity. Thus, the parameters measured and observed are summarized in Table 2.

Following the commencement of the experiment, a period of at least 48 hours elapsed before meticulously extracting 0.2 ml of water from each of the five treatments using a micropipette. Petri dishes corresponding to each treatment and the insect were randomly selected. The collected water samples were then inoculated onto dishes containing an agar culture medium to facilitate the growth of microorganisms. The dishes were incubated at 27°C for 48 hours. The identification of taxa of components of the biofilm was made at a high taxa level because the objective of this work was mainly about the ecological role and not taxonomic. In addition, it is essential to point out that in none of the treatments can the chemical substances ultimately inhibit the development of microorganisms. The dishes were observed under an optical microscope to determine the microorganism components present in each treatment. The bacteria identification was performed using standard methods like Gram Stain and culture medium (**PCA**) and four selective media (Mac Conkey culture Caldo, Agar Manitol-Salado, EC media, and Agar with Eosina and Methylene Blue) for the identification of groups of bacteria (more detail in Supplementary Information). Figure 3 summarizes the experimental methodology.

Table 2. Parameters were measured and observed in the treatments throughout the experiment.

Parameter	Stage	Description
Water-color change	No change (0)	Transparent
	Change (1)	Light yellowish gray color
Water turbidity	No turbidity (0)	Transparent
	Low turbidity (1)	Few suspended particles
	Medium turbidity (2)	Suspended particles partially impede visibility
	High Turbidity (3)	Suspended particles partially impede visibility
Biofilm	Absent (0)	No biofilm is observed
	Present (1)	Biofilm is observed
Density of biofilm	No density (0)	Insects are completely visible
	Low density (1)	The cover allows slight observation of the insect
	Medium density (2)	The cover hardly allows observation of the insect
	High density (3)	The cover does not allow observation of the insect
Disarticulation of aquatic insect	No disarticulate (0)	Complete corpse
	Disarticulate (1)	Loss of at least some element

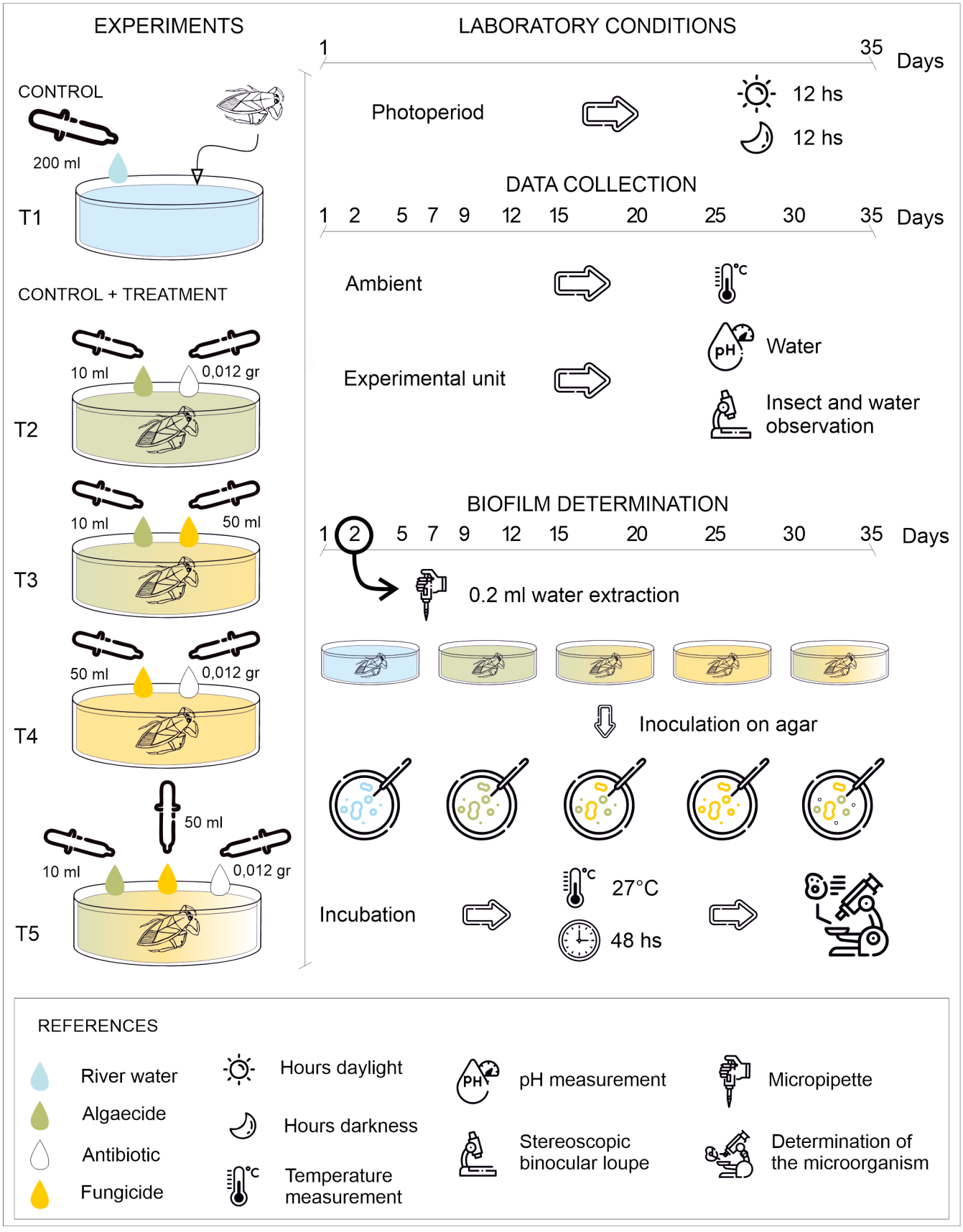

Figure 3. Schematic representations of the experimental models illustrating the methodology.

Five samples from each treatment were dehydrated first. They were metalized with gold for observation with **SEM** (Scanning Electron microscope LEO 1450VP) at the Laboratory of Electronic Microscopy and Microanalysis (**LABMEM**, Universidad Nacional de San Luis). The images were obtained through secondary electrons. Throughout the 35 days of the experiment, the microorganism components of each experimental unit were identified using the methodology explained.

RESULTS

The recorded air temperature had a maximum value of 36º C on days 5 and 7, and the minimum was 16º C on day 20. The average temperature recorded was 29.9° C with a standard deviation of b ± 2.63. Water and air temperatures in the laboratory show lower variation, with an air temperature maximum of 34 ºC on day 7 and a minimum of 24 ºC on day 35. Water temperature measured in all treatments shows a similar curve with little variations between 0.5 and 1º C. Regarding pH measurements, more significant variations were observed between T1 (control) and T5 (combined), with values beyond neutral to alkaline. The rest of the treatments show slight variation in this subject. Average values of pH and standard deviation in each treatment were: T1 (6.95 ± 0.60), T2 (6.90 ± 0.32), T3 (7.1 ± 0.21), T4 (7.05 ± 0.16), T5 (7.20 ± 0.35). The growth of these microorganisms occurs naturally in treatment 1 (T l), which has no inhibitor at all. The rest of the treatments were prepared, including combinations of different inhibitors that allow for evaluating the formation time and the other components' activity over the insect bodies.

In T1 (Control), one of the parameters recorded was the water-color change recorded for the first time on day 7, with posterior records on days 15, 30, and 35. The increase in the water turbidity was recorded from day 5 (value 2) with a peak (value 3) on day 7 with a decrease to value one during the rest of the days. Biofilm occurrence is documented starting from day 2 and remains constant in the subsequent days, although displaying some fluctuation in density, ranging between values 2 and 3 throughout the experiment (Figure 4). The microorganisms recorded in the biofilm in this treatment include bacteria, fungi, and algae that were observed using an optical microscope (Table 3). With much better resolution in the SEM, it was possible to determine the presence of abundant bacteria (bacillus type) and algae, mainly diatoms (Table 3). Biofilm bacterial components were determined to be Staphylococcaceae and Enterobacteriaceae. Disarticulation of the T1 insects was observed from day 7 onward (Figure 4).

In T2, the treatment with algaecide and antibiotic, the water-color change was recorded on days 20, 25, and 30, with the water turbidity increasing from value one on day 9 to value two on days 25 and 35 and with a maximum of value three on day 30. Biofilm occurrence was observed on day 2, and, except for day 5, it was kept until the end of the experiment (Figure 5). About the density of the biofilm, it shows values from 1 on days 2, 7, and 9 to 3 on days 12 to 35. The microorganisms recorded included fungi hyphae, mycelia, and spores detected under an optic microscope. Under the SEM, it was also possible to see reproductive structures or conidia (Table 3). In this treatment, no disarticulated insect bodies were recorded at any sampling point (Figure 5).

In T3, the treatment with algaecide and fungicide, a water-color change was recorded on days 7, 12, and 25, with water turbidity values of 1 from day 7 to 30 and a value of 2 on day 35. Biofilm occurred from day 2 until the end of the experiment, with an exception on day 5 in the random dishes selected. The biofilm density was two between days 2 and 25 and 3 on days 30 and 35. The biofilm microorganism components include bacteria, algae, and fungi that were determined with observation through an optic microscope. Using SEM, it was possible to see algae, mostly diatoms (Table 3, Figure 6). Biofilm bacteria components recognized were Staphylococcaceae, and the fungi were *Aspergillus niger*. In T3, the disarticulation of the insect bodies occurred from day 12, except on day 20, in the random dishes selected (Figure 6).

Treatment 4, or T4, which involved using antibiotics and fungicides, exhibited no watercolor changes throughout the experiment. Nevertheless, there were fluctuations in water turbidity, transitioning from values of 1 on days 7 and 9 to 2 from days 12 to 35. The biofilm occurred for the first time on day 2 and showed persistence until the experiment ended, increasing in density from values one on day 2 to values two on days 5 to 9 and 3 until day 35. The microorganism components observed under the optical microscope in this treatment were algae and bacteria of coccoid type (Table 3). Abundant diatoms and coccoid bacteria were recognized under the SEM (Figure 7). Biofilm bacterial components identified were eubacteria Staphylococcaceae. This treatment recorded disarticulation of the insect body just on day 30 (Figure 7).

Finally, treatment 5 (T5), the combined treatment, showed water-color changes on days 9, 15, 25, and 30, with water turbidity values one from day 9 through the end of the experiment. Biofilm appears for the first time on day seven and extends until day 25. The biofilm density recorded values from 2 on days 7 and 20, 1 between days 9 and 15, and 3 on days 25. The biofilm components included bacteria, according to the observation in optic microscopic. Meanwhile, according to observations in SEM, the biofilm components had algae from the diatom, bacteria, and bacillus types (Table 3, Figure 8)—specifically, the bacteria belonging to Enterobacteriaceae. The combined treatment with algaecide, antibiotics, and fungicide resulted in the disarticulation of the insect body only on days 20 and 25 of the experiment (Figure 8).

DISCUSSION

Biofilm

Experimental work evaluating the different aspects of forming a microbial mat/biofilm was generally focused on marine organisms and environments (*e.g.*, Raff *et al.*, 2014; Butler *et al.*, 2015; Klompmaker *et al.*, 2017). Works about freshwater environments, especially lakes, are scarce

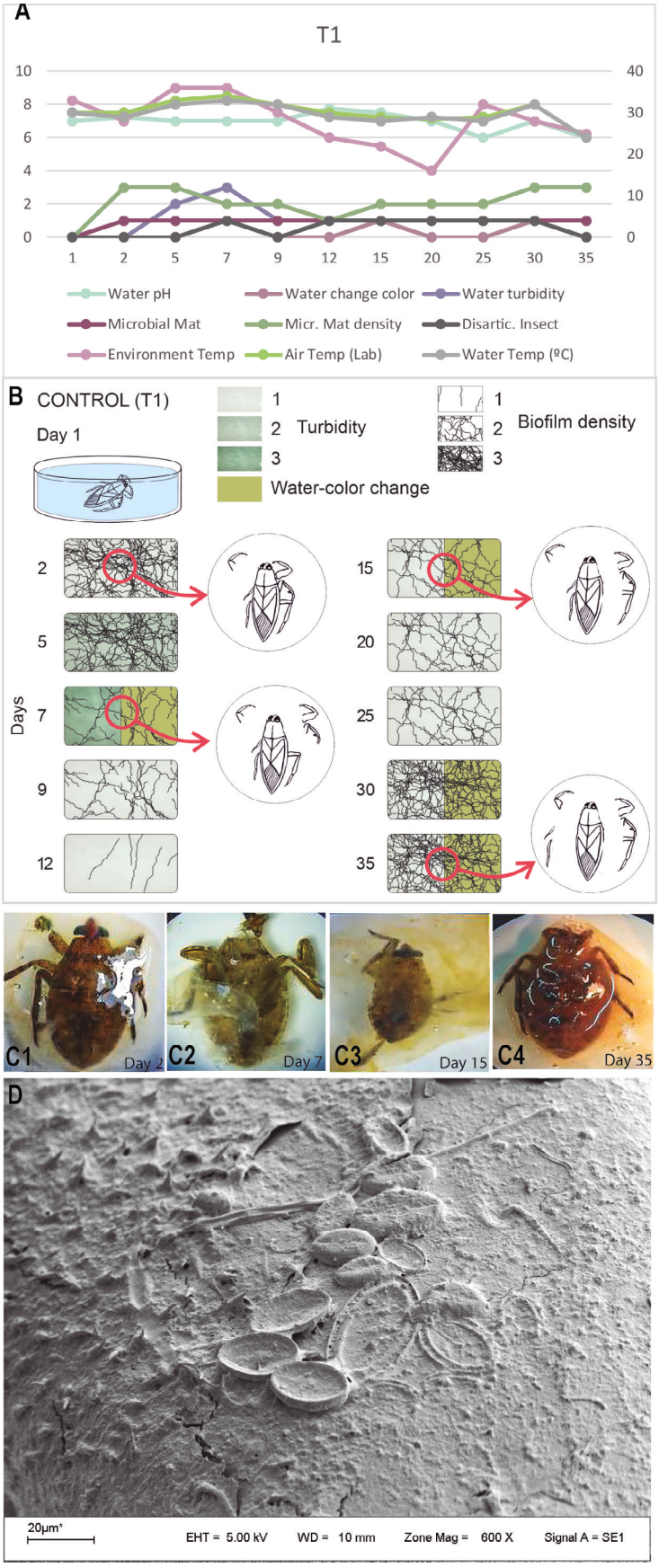

Figure 4. Treatment 1 (Control). **A,** parameters measured and observed during the 35 days of the experiment on T1. **B**, visual model schemes illustrating the dynamic process of biofilm formation across T1. **C1**–**4**, photographs of experimental units on days 2, 7, 15, and 35. **D**, microorganisms were recorded in each treatment observed in SEM.

Table 3. Microorganisms were recorded by Optical Microscopy (**OM**) and Scanning Electron Microscopy (**SEM**) in each treatment.

Treatment	OM	SEM	Bacterial identification
T1	Bacteria Fungi Algae	Bacteria bacilli Algae Superclas Diatomea	Staphylococcaceae Enterobacteriaceae
T2	Fungi spores	Dense mycelium of hyphae Conidia (reproductive structure)	
T3	Mobil Bacteria Algae Fungi	Algae Superclas Diatomea	Staphylococcaceae
T4	Algae Coccoid Bacteria	Algae Superclas Diatomea Coccoid Bacteria	Staphylococcaceae
T5	Mobil Bacteria	Algae Superclas Diatomea Bacteria bacilli	Enterobacteriaceae

and mainly focused on vertebrates (fish and amphibians) (*e.g*., Iniesto *et al*., 2013, 2015, 2016, 2020). In particular, the formation timing of the biofilm principal components in aquatic insects and freshwater environments was not previously analyzed in detail. Like observations made by other authors (Iniesto *et al*., 2016, 2020; Tian *et al*., 2020), the taphonomic experiment in this study validated the presence of various organisms contributing to biofilm formation. The first occurrence of microbial biofilm was recorded on day 2 in most treatments. Treatment 5 (T5) recorded the biofilm on day 7, probably due to the combination of inhibitors. The early occurrence of a superficial biofilm was documented by Butler *et al*. (2015) in marine environments and observed by Martínez- Delclós & Martinell (1993) in a taphonomic study, where it described the formation of biofilms, including fungi and/or algae around the insects that floated in freshwater bodies.

The microorganisms present in the biofilm from the treatments comprise fungi of the species *Aspergillus niger* and others from the Zygomycota group. The algae recorded are in part Bacillariophyceae and the Zygnematales. The bacteria are mostly Eubacteria belonging to Enterobacteriaceae, like *Escherichia coli* and Staphylococcaceae. A biofilm was developed based on the structure and microorganism components in the five treatments studied. The characteristic laminar stratification of the microbial mats was not observed in any of the treatments, different from the documented by previous authors (Iniesto *et al*., 2013, 2015, 2016), where these mats were found well developed, maybe because of the duration of the experiments, that extends in some cases for years.

Several authors sustained that the physicochemical conditions (*e.g*., pH, alkalinity, redox potential, salinity, oxygen concentration, light, temperature, water content, and velocity of the current) are the potential factors that promote or inhibit the growth of microorganisms in these systems (*e.g*., Decho, 2000; Stolz, 2000; Iniesto *et al*., 2016). While no deliberate parameter variations were introduced during the experimentation, we meticulously analyzed the naturally occurring environmental fluctuations. Throughout the present study, the observed variation in measured physicochemical variables revealed no direct relationship between the development of the biofilm. The minor variations in the pH and temperature in the experimental units did not show a direct link with the variation in the occurrence and density of the biofilm. Unfortunately, the pH variation inside the biofilm cannot be measured. Because of that, we cannot corroborate Iniesto *et al*.'s previous observations (2016) about inner acidification and alkaline conditions outside the structure. In the experiment in this paper, only two occasions were recorded pH 6 (in the T1, day 25, and the T2, day 12) with a remarkable change in the biofilm density only in the T2. Concerning water temperature, only small fluctuations were recorded that show no relation to the occurrence or density of the biofilm. Because the air temperature in the laboratory showed similar values to the water temperature recorded in the experiment, its influence on changes in the characteristics of the biofilm was not considered.

Based on the data obtained from this experiment, the significance of changes in physicochemical parameters as factors that promote or inhibit the growth of microorganisms cannot be confirmed or denied. Further research may be needed to understand better the specific physicochemical parameters that impact microbial growth in this context.

Aquatic insect preservation

There are studies both in classical and experimental taphonomy that focus on insects (*e.g*., Martínez-Delclós & Martinell, 1993; Martínez- Delclós *et al*., 2004, Mancuso *et al*., 2007; Smith, 2012; Wang *et al*., 2013; Greenwalt *et al*., 2015; Iniesto *et al*., 2016, 2020; Osés *et al*., 2016; Dias & Carvalho, 2022). Remarkably, aquatic insects were not deeply evaluated in this perspective. Therefore, we can consider the biostratinomic processes documented for terrestrial insects in freshwater systems comparable to those experienced by aquatic insects upon entering the water body.

Sierra (2019) reviewed the aquatic insect collection from the La Cantera Formation and revealed a record of 45%

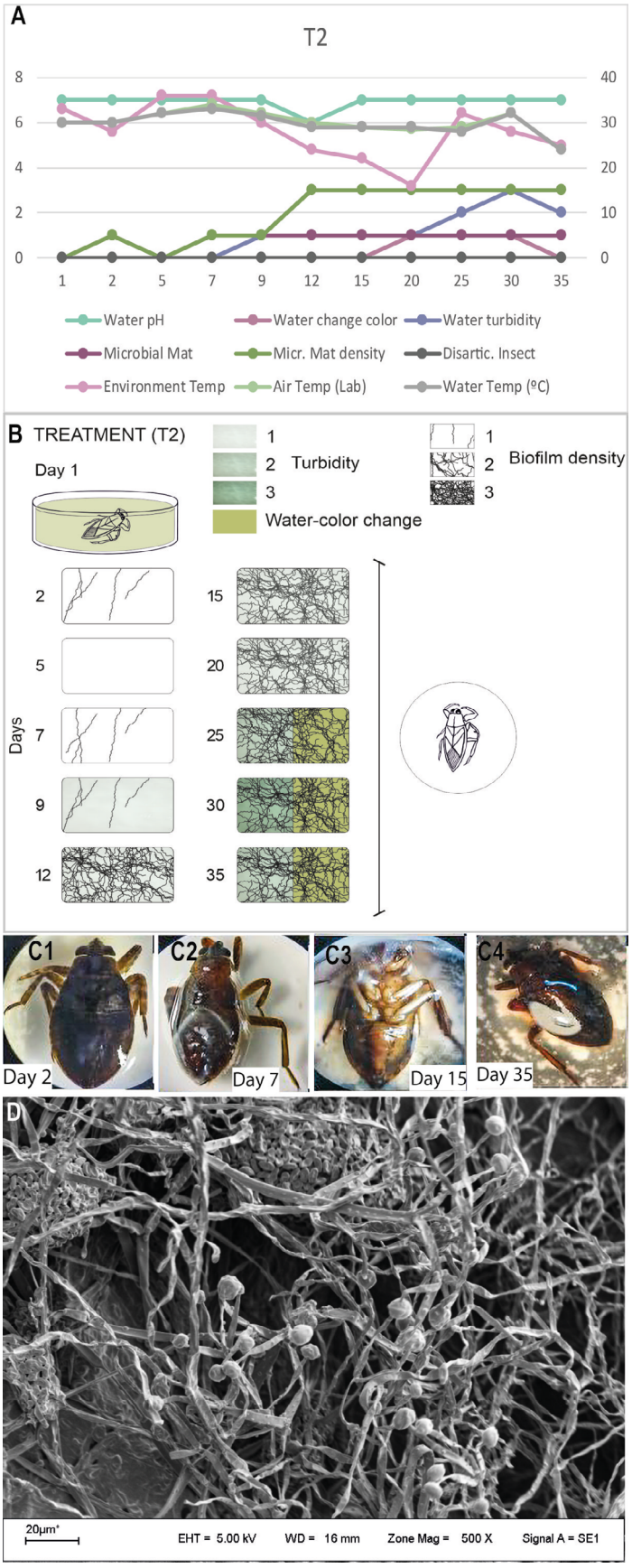

Figure 5. Treatment 2 (algaecide + antibiotic). **A**, parameters measured and observed during the 35 days of the experiment on T2. **B**, visual model schemes illustrating the dynamic process of biofilm formation across T2. **C1**–**4**, photographs of experimental units on days 2, 7, 15, and 35. **D**, microorganisms were recorded in each treatment observed in SEM.

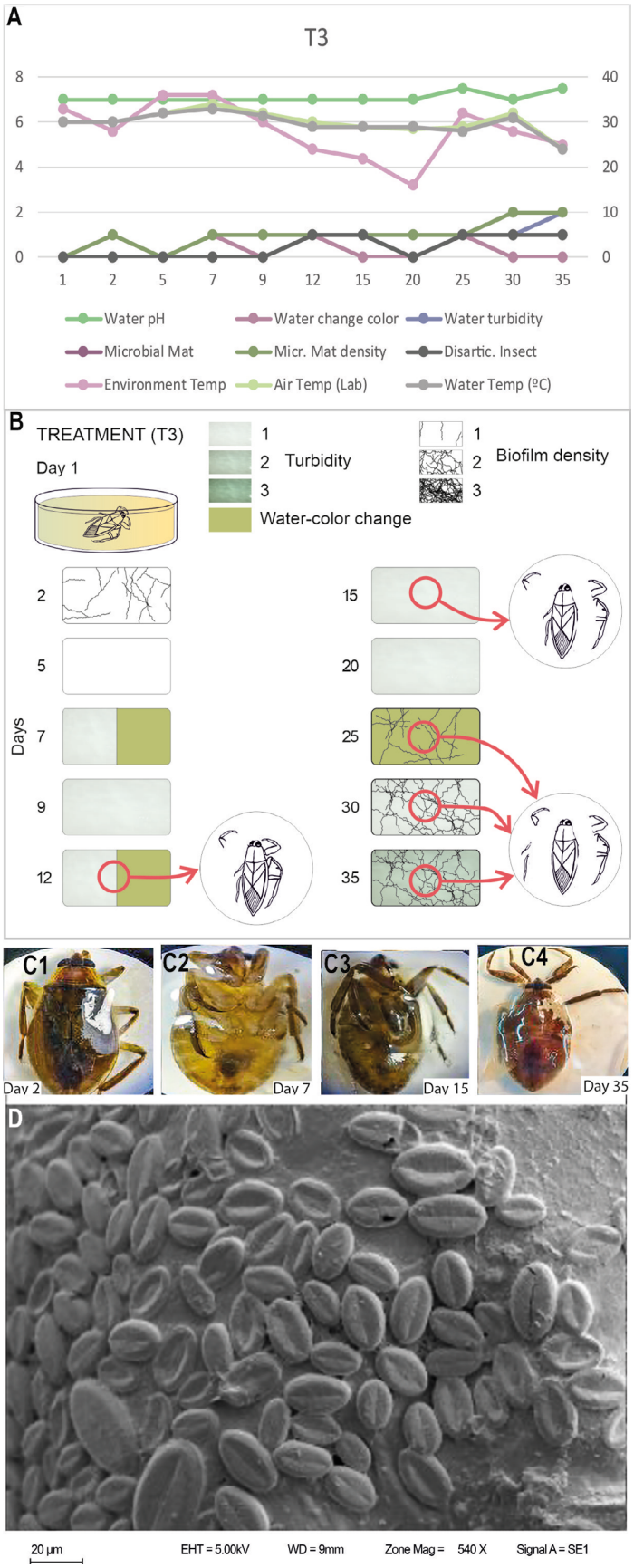

Figure 6. Treatment 3 (algaecide + fungicide). **A**, parameters measured and observed during the 35 days of the experiment on T3. **B**, visual model schemes illustrating the dynamic process of biofilm formation across T3. **C1**–**4**, photographs of experimental units on days 2, 7, 15, and 35. **D**, microorganisms were recorded in each treatment observed in SEM.

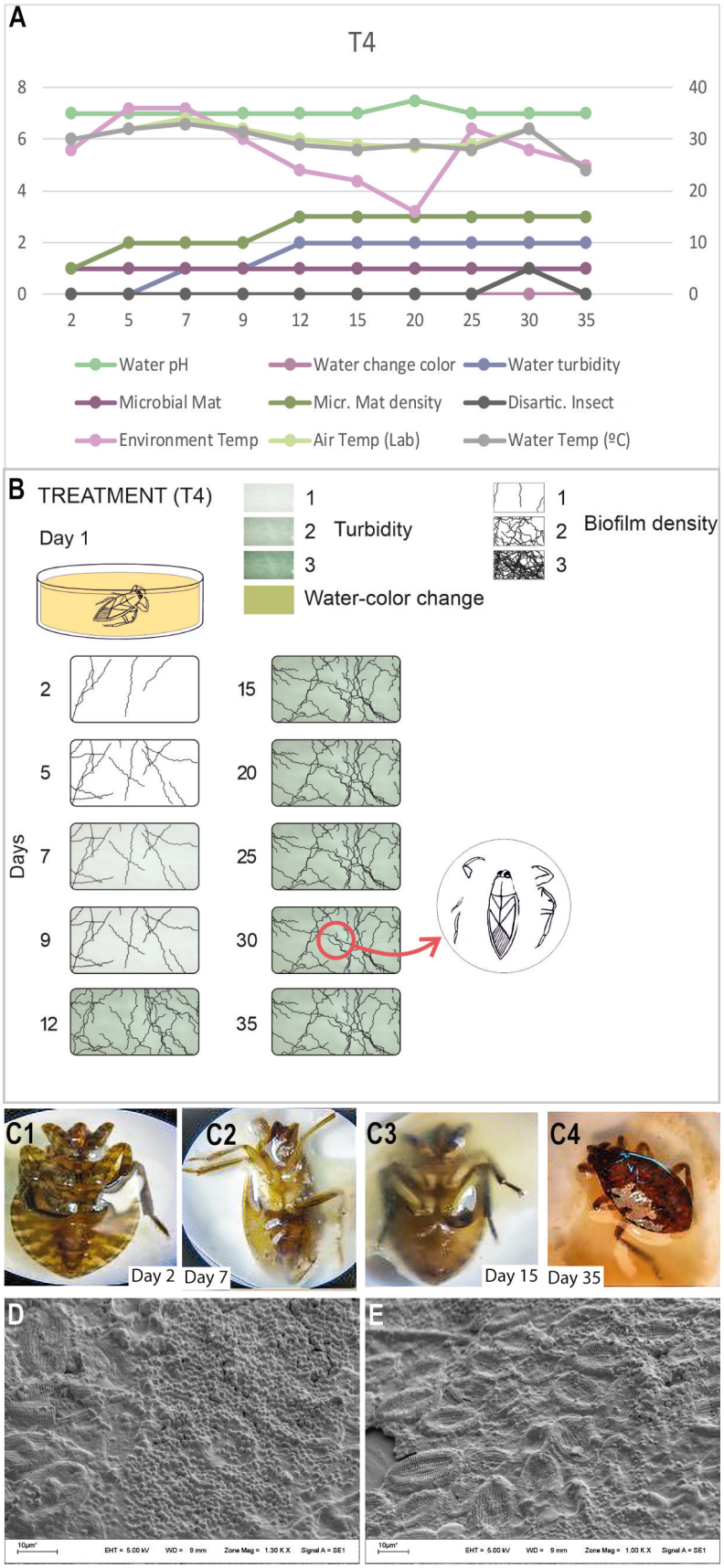

Figure 7. Treatment 4 (antibiotic + fungicide). **A**, parameters measured and observed during the 35 days of the experiment on T4. **B**, visual model schemes illustrating the dynamic process of biofilm formation across T4. **C1**–**4**, photographs of experimental units on days 2, 7, 15, and 35. **D-E**, microorganisms were recorded in each treatment observed in SEM.

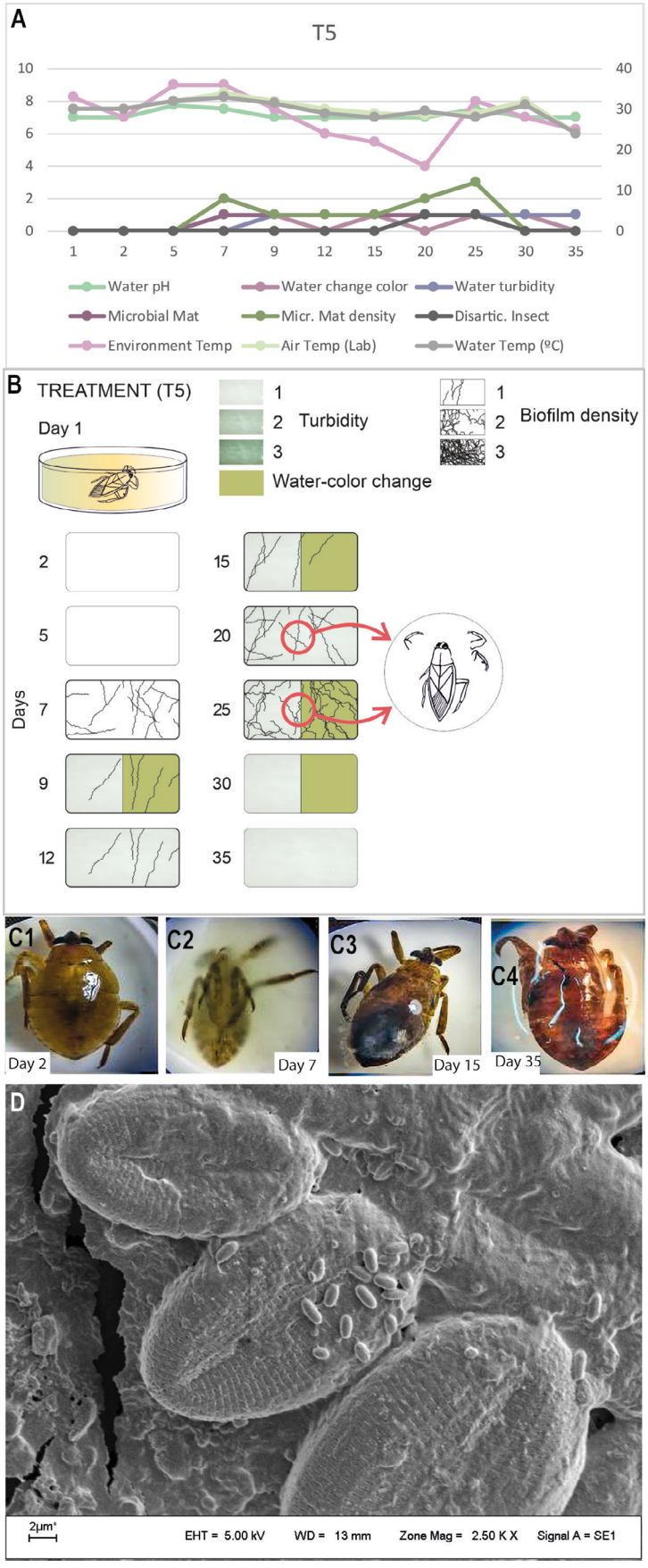

Figure 8. Treatment 5 (combined). **A**, parameters measured and observed during the 35 days of the experiment on T5. **B**, visual model schemes illustrating the dynamic process of biofilm formation across T5. **C1**–**4**, photographs of experimental units on days 2, 7, 15, and 35. **D**, microorganisms were recorded in each treatment observed in SEM.

of adult individuals and 55% of exuvius, and only 5% of them were found articulated, whereas the rest were partially articulated. There is no record of isolated elements from aquatic insects. Disarticulation of the head was recorded in 90% of the adults and 81% of the exuvius since the loss of the appendix was 71% in adults and 80% in exuvius. The wings were disarticulated in 80% of adults and 100% of exuvius. Significantly different values are observed in disarticulating the structures of hydrofuge hairs in the ventral part of the abdomen, with losses of 93% in adults and 7% in exuvius.

Regarding biofilm development, in the taphonomic study of Martínez-Delclós & Martinell (1993), they described the growth of fungi and algae surrounding the corpses of terrestrial insects while floating in the water column. Furthermore, they proposed that this phenomenon could potentially enhance the relative weight of the remains, causing them to sink to the bottom of the water body. The presence of biofilm was consistently documented as early as day 2 in most treatments of the present taphonomic experiment, except for T5 (combined treatment), which would suggest potential biofilm development even when the insect body remained suspended in the water column.

Articulated fossil aquatic insects from the La Cantera Formation (Early Cretaceous), as the Crato Formation from Brazil (*e.g*., Bezerra *et al*., 2021), raises questions about the involvement of biofilm in insect preservation. According to classical and experimental taphonomy studies, when aquatic insects die, their carcasses do not penetrate the surface tension and remain on the surface or epilimnion for some time, favoring the decomposition process that promotes disarticulation. Therefore, the different treatments showed that the start of the disarticulation process varies. The natural conditions, represented by T1 (control), show disarticulation for the first time on day 7. In T3, containing inhibitors for algae and fungi, disarticulation occurs a few days later and is a possible result of bacterial activity. Although endogenous microorganisms were not evaluated in the present work, they can be inhibited after frozen insects. The results of T3 could suggest that this treatment without bacterial inhibitors could favor the growth of internal and external bacteria that then produce the disarticulation observed. However, other analyses revealed that the microbial mats/biofilms, primarily consisting of bacteria, served as mediators of tissue mineralization (*e.g*., Greenwalt *et al*., 2015; Osés *et al*., 2016; Varejão *et al*., 2019; Bezerra *et al*., 2020; Dias & Carvalho, 2022).

Treatment T2, which includes inhibitors of algae and bacteria and shows the growth of fungi, disarticulation was not observed during the 35 days of the whole extension of the experiment. T4, including inhibitors of fungi and bacteria, predominantly exhibited the growth of various algae, with disarticulation observed only on day 30. This result agrees with the observations recorded by Martínez-Delclós & Martinell (1993), which followed the development of fungi and algae around the corpses of insects floating, acting as a stop for the fragmentation and disarticulation and keeping body parts united. While it may seem contradictory, fungi might prefer preservation within aquatic insects. Despite fungi primarily serving as the primary decomposers in terrestrial environments, the presence of fungi and/or algae on aquatic insects could function more as a protective barrier and covering than a catalyst for tissue mineralization.

The potential disarticulation of the insect body, when they reach the freshwater body bottom, is essentially related to the presence of benthic organisms (crustaceous, mollusks, or fishes) and/or to the bottom currents that can generate fragmentation of the remains. In the laboratory, it was observed that without biological or physical agents that disturb their bodies, insects could maintain even until a year without disarticulation in quiet sedimentary environments (Martínez-Delclós & Martinell, 1993). However, certain organisms like chitinophaga bacteria favor the disarticulation mainly in the joint areas where cuticles are less thick. In a study about cicadas, Wang *et al*. (2013) observed that 20% of the specimens presented disarticulation of the heads after 14 days. On the contrary, during the 35 days of our experiment, the disarticulation of the head was not observed, but most disarticulation occurred between the appendix and the body. The results of the experiments done in this work suggested that in the treatments T2 and T4, the degradation of insect corpses was less favored by the growth of fungi in one case and algae in the other. Based on these results, it is possible to propose that biofilms composed of algae and/or fungi will favor the preservation of the articulated body of aquatic insects once they get to the bottom of a lake by the potential protection from other biotic or abiotic agents and because of their cohesive activity. Indeed, an alternative hypothesis is that inhibition of the bacterial component may significantly affect morphological integrity, even without implicating a protective role for other biological components of the biofilm.

Numerous studies on insects highlight the role of microbial mats in enhancing fossil preservation (*e.g*., Greenwalt *et al*., 2015; Osés *et al*., 2016; Varejão *et al*., 2019; Bezerra *et al*., 2020; Dias & Carvalho, 2022). Moreover, most of them proposed that biofilm formed mainly by bacteria is the catalyst for tissue mineralization. Therefore, different precipitation minerals favor insect preservation (Osés *et al*., 2016; Varejão *et al*., 2019; Bezerra *et al*., 2020; Dias & Carvalho, 2022). In the T3, the developed bacteria did not exhibit decomposition and disarticulation protection because of their joint activity with endogenous bacteria or less cohesive action.

The fossil aquatic insect record from the La Cantera Formation shows similar proportions between exuvius and body parts. Only 5% of the remains are fully articulated; the rest were partially articulated. Although incomplete, the high proportions of articulated elements suggested some factors acting in the past, such as biofilm formation, which prevents disarticulation of the structures. In particular, the exuvius could suffer disarticulation during the change of the insect cuticle, as Janello (2012) suggested based on a flotation experiment. In these observations on living Heteroptera insects, the thorax opens dorsally during ecdysis, and the exuvius keeps floating on the surface for more than 30 days because of their low specific weight. In natural environments, water perturbations allow them to sink and reach the bottom.

In this experiment, the exuvius were found in a ventrally floating position, which coincided with the present record from the La Cantera Formation. The disarticulation of the head and appendix was also registered in high proportions in the observations of Janello (2012) as recorded in the La Cantera Formation. The Crato Formation insects reported a very similar condition, where the insects are articulated with exceptional preservation (*e.g*., Osés *et al*., 2016; Varejão *et al*., 2019; Bezerra *et al*., 2020; Dias & Carvalho, 2022).

The Crato Formation (Aptian of the Araripe Basin, Brazil) consists of lithographic limestones that preserved insect fossils with a high degree of morphological fidelity to external and internal anatomical features (Dias & Carvalho, 2022). In the Crato insect record, in contrast with the La Cantera, it is very well-documented that bacterial biofilms played a role in tissue mineralization (Osés *et al*., 2016; Varejão *et al*., 2019; Bezerra *et al*., 2020; Dias & Carvalho, 2022). The Crato sedimentary context, with laminated carbonate layers attributed to chemical precipitation, interbedded with siliciclastic sediments (claystone and sandstone) (Heimhofer *et al*., 2010), favored the mineralization mediated by microbial mats. On the other hand, the La Cantera sedimentary context, with exclusively siliciclastic sediments (claystone and sandstone), only offered indirect evidence of microbial activity, such as wrinkles, syneresis cracks, and kidney structures (Arcucci *et al*., 2015; Castillo Elías, 2016).

The experiments presented in this article and the patterns observed in the Cretaceous La Cantera Formation aquatic insect record are consistent with the development of biofilms in early *post-mortem* stages. They are most probably developed on the exuvius and the body during the flotation period. The taphonomic experimental reflected that the biofilm was composed initially of algae and fungi covering the corpses, as was documented by Martínez-Delclós & Martinell (1993), proposing that it could have a dual function, acting cohesively and avoiding fragmentation and disarticulation and, on the other side, increasing the relative weight helping in the sinking of the corpse to reach the bottom of the water column. The alternative hypothesis regarding the role of bacteria could provide a deeper understanding of the mechanisms involved in corpse preservation and the interactions among various microbial components. Further exploration of this hypothesis could offer valuable insights into the dynamics of microbial communities and their effects on paleontological deposits.

Moreover, the experimental analyses conducted in this study suggest that the observations could potentially be applied to the exceptional preservation of insects in quiet aquatic environments. By extrapolating the findings and using them to similar environments, researchers may better understand the processes involved in preserving insect specimens. This could have significant implications for paleontological research, particularly in understanding the preservation of delicate organisms in specific ecological settings.

CONCLUSIONS

The taphonomic experiment on aquatic insects evidences that microorganisms form a biofilm to cover the corpses, protecting the organic remains or diminishing their degradation. The microorganisms identified during the experiments developed and components of the biofilm communities were fungi (*Aspergillus niger* and Zygomycota), algae (Bacillariophyceae and Zygnematales), and representatives of Eubacteria (Staphylococcaceae and Enterobacteriaceae).

The treatments T2 and T4 reveal that the degradation of aquatic insect corpses was less pronounced, a phenomenon attributed to the flourishing growth of fungi and algae within the biofilm, in agreement with observations made by Martínez-Delclós & Martinell (1993). These microorganisms play a pivotal role in the selective preservation of aquatic insect fossil remains, as exemplified by findings in the Crato Formation.

Contrastingly, in the La Cantera Formation from the Early Cretaceous period, where evidence of bacteria mats is only indirectly discernible and not closely associated with insects, the well-preserved articulated insect record could be attributed to the presence of fungi and algae biofilm. Therefore, the present research highlights the importance of understanding the role of microorganisms in preserving fossils and contributes to our understanding of paleontological processes.

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