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ORIGINAL ARTICLE

Environmental and cultured cyanobacteria as sources of *Aedes aegypti* larvicides

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Abstract

In tropical countries, the control of the mosquito *Aedes aegypti* is a public health priority due to its role as a vector of important viral diseases. Marine cyanobacteria are recognized as abundant sources of bioactive compounds, and they constitute a potential source of insecticides useful for controlling mosquito populations and preventing epidemic outbreaks. We collected 30 benthic cyanobacterial mats in Providencia and Rosario islands (in the Colombian Caribbean) belonging to the genera *Phormidium*, *Symploca*, *Oscillatoria*, *Lyngbya*, *Pseudoanabaena*, *Leptolyngbya*, *Moorea*, and *Dapis*. Fractions of organic extracts from the most abundant environmental samples were evaluated in three bioassays, assessing (i) larvicidal activity against *A. aegypti*, (ii) toxicity against the brine shrimp (*Artemia salina*) nauplii, and (iii) acetylcholinesterase inhibition. Non-polar fractions exhibited larvicidal activity. The polar fraction from one *Dapis pleuosa* extract showed larvicidal activity without being toxic against *A. salina* nauplii. Extracts from *Moorea producens* exhibited the greatest toxicity against *A. aegypti* larvae and *A. salina* nauplii. From 23 cultured cyanobacterial samples, only five grew under laboratory conditions and produced enough biomass to yield organic extracts. Of these, three extracts showed strong larvicidal activity, but only the extract from *Phormidium tenue* showed reduced toxicity against *A. salina* nauplii. We detected variation among the chemical profiles and larvicidal activity of cyanobacterial consortia depending on sites and dates of collection. Our findings suggest that despite variation in chemical profiles, extracts of marine benthic cyanobacteria can be further developed as effective control agents against insect vectors, in their larval stages. The culture of marine benthic cyanobacteria needs to be further explored to provide enough biomass leading to the identification of bioactive compounds with public health applications.

Keywords: Cyanobacteria; *Aedes aegypti*; *Artemia salina*; larvicidal activity; acetylcholinesterase inhibitors, cyanobacterial culture.

Introduction

Mosquitoes (Diptera: Culicidae) are important vectors of several viral diseases and are a topic of public health concern in tropical countries. Diseases such as dengue, chikungunya, zika, and yellow fever are mainly transmitted via

the mosquito *Aedes aegypti* [1]. These four diseases have a large impact on public health systems due to their high incidence, increasing infection rates, and increasing treatment costs. In Latin America, the recent outbreaks of zika and chikungunya exemplify the gravity of these diseases [2, 3]. Efforts to control these diseases have focused on targeting either their underlying viruses or their vectors [4]. Vector control via insecticide applications constitutes a widespread alternative to avoid the growth of large mosquito populations. However, insecticide-associated environmental and health impacts, together with the development of insecticide resistance in many mosquitoes, including *A. aegypti*, [5], call for new sources of compounds to control mosquito populations.

Cyanobacteria are microorganisms that thrive in a great diversity of aquatic and terrestrial environments. Their survival is linked to their broad array of metabolic products associated with defensive and competitive (algaecides) functions. These metabolites include, but are not limited to, cyclic and linear peptides, guanidines, purines, lipids, macrolides [6, 7]. Recently, some studies have evaluated the potential of cyanobacteria and their metabolites towards the development and production of application in the areas of biofuels, anticancer agents, and pesticides [6]. Marine cyanobacteria-derived compounds, such as unsaturated fatty acids and sulfated glycolipids, have revealed promising activities against larvae of the mosquito species *A. aegypti* and *Aedes albopictus* [8, 9]. Hence, cyanobacterial metabolites represent a potential source of compounds that may be used as an alternative for controlling *Aedes larvae*.

One of the main challenges when utilizing marine natural products in the pharmaceutical and agrochemical industries is their supply [10]. This challenge escalates when promising compounds are derived from marine organisms with low population densities. With cyanobacteria, however, this may not be the case. The increase, recurrence, and persistence of large cyanobacterial blooms, as a consequence of excessive nutrient input into coastal waters and global warming [11], propitiates the obtention of sufficient amounts of cyanobacterial compounds with biological activity. Also, the culture of marine microorganisms is a feasible approach to overcome supply limitations [12-15]. Most chemical studies of cyanobacteria have been thus far performed on environmental samples [16, 17]. Therefore, the culture of cyanobacteria, although challenging because these organisms do not easily respond to *in vitro* conditions [18-20], could help understanding bloom dynamics and provide enough biomass to obtain promising bioactive compounds [21, 22].

As a continuation of our studies on the chemistry and potential use of cyanobacteria from the Colombian Caribbean [23], in this work we explored the insecticidal activity of polar and non-polar extracts of marine cyanobacteria against *A. aegypti* larvae. We assessed the potential toxicity of these extracts with assays involving *Artemia salina* nauplii. Lastly, we investigated a possible mechanism of action of these extracts as acetylcholinesterase (AChE) inhibitors. All our evaluated extracts were obtained from environmental samples and from cultured cyanobacterial mats **(Fig. 1)** that yielded enough biomass to perform the assays.

Materials and methods

Cyanobacterial mat collection

Samples of benthic cyanobacterial mats were collected in waters off the coasts of Old Providence Island and the Archipelago of Islas del Rosario, Colombia. Old Providence Island is part of the Archipelago of San Andrés, Old Providence, and Santa Catalina, a group of oceanic islands, submerged banks, and atolls located approximately 300 km off the coast of Nicaragua [25, 26]. The shallow reef complex in Old Providence has a total area of 285 km^2 and is characterized by the development of different coral formations [27-29]. The archipelago of Islas del Rosario (Departamento de Bolívar) consists of a group of islands and low coral reefs of recent origin. In Old Providence, samples were collected at Felipe's Place (13°18'-13°24' N; 81°19-81°25' W), at depths between 10-20 m, and in Islas del Rosario sampling

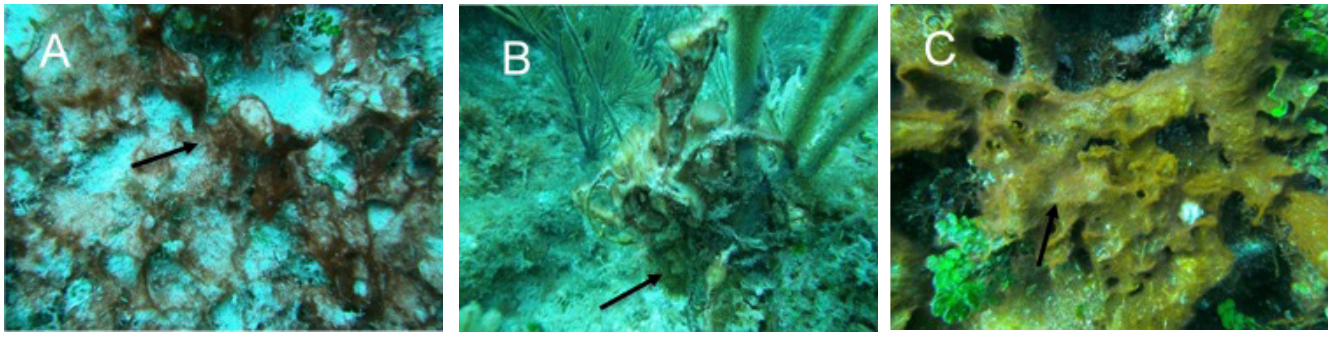

Figure 1. Cyanobacterial mats photographs taken at collection; **A)** Filamentous mat growing on a coral skeleton, Islas del Rosario 2016 (10-12 m deep), **B)** Green turf mat growing on an octocoral; the mat causes necrosis and partial mortality, Providencia 2015 (20 m deep), **C)** Filamentous mat growing on algae, Islas del Rosario 2016 (10-12 m deep).

sites were Ministerio (10°11'06" N, 75°43'55" W), at depths between 10-20 m and Pavitos (10°10'30" N, 75°46'17" W), at a depth between 20-25 m. All sites are well-developed fringing reefs, with a predominance of patches with low coral diversity that determine their ecology [30].

Immersions were performed at each collection site from June to August 2009, 2010, 2015, and 2016 to detach and collect cyanobacterial mats from sand and live substrates. During the sampling months, the northeast winds intensify, temporarily inhibiting the rainy season [24]. Upon collection, cyanobacterial mats were deposited in mesh bags, keeping track of site and depth of collection. The collected material was cleaned from debris and kept frozen (–4°C) until chemical studies were performed. Small mat samples (50 cc) were stored in 10 % formalin in seawater to perform morphometric analyses. Vouchers for each cyanobacterial mat were deposited at the IBUN (Instituto de Biotecnología-Universidad Nacional de Colombia) collection.

Identification of cyanobacterial samples

Cyanobacterial identification was performed on morphological characters. To identify the different cyanobacterial morphotypes, at least three portions from each sample were observed under a Nikon optical microscope connected to a digital camera. The captured images were analyzed using NIS-Elements Br 2.30 Nikon Imaging Software, which allowed performing measurements of each morphological character under several magnifications up to 100X. Distinctive characters included: trichome length, width, and number of ramifications; the presence/absence of specialized cells such as heterocysts or akinetes; presence of a facultative (fine to stratified) sheath; cell length to width ratio; the presence of calyptrae; the shape of apical cells; intercellular constrictions; and presence of mucilaginous cases [31-33].

General experimental information

Larvae of *A. aegypti* (Rockefeller strain) were donated by Fernando Noriega from Florida International University, Miami, USA. Cysts of the brine shrimp *A. salina* were purchased from Artemia International (USA). Reagents for the acetylcholinesterase inhibition assay, acetylcholinesterase (EC. 3.1.1.7) from *Electrophorus electricus* (type VI-S 500 A), bovine serum albumin (BSA), phosphate buffer 0.1 M, 1-naphthyl acetate, fast blue salt, and Malathion and 4,4'-DDT (used as positive controls in the larvicidal assay) were purchased from Sigma-Aldrich (Darmstadt, Germany).

Chemical separation analyses were performed with the following analytical quality solvents: MeOH, DCM, and BuOH (Merck, Darmstadt, Germany).

Thin layer chromatography was performed on aluminum plates precoated with silica gel 60F254 (Merck, Darmstadt, Germany). HPLC-ELSD analyses were performed on a Thermo Dionex ultimate 3000 system, coupled to an ELSD Sedex 85 detector (Sedere, France) with a gain of 10 for the ELSD detector and a temperature of 80 °C.

Extraction and chemical partitions of environmental cyanobacteria samples

The extraction step on each sample was performed using a DCM/MeOH (1:1) mixture. Solvents were removed under reduced pressure to yield a crude extract. Each crude extract was weighed and then resuspended in water and mixed with dichloromethane to yield the DCM (FD) fraction. The water layer was then extracted with butanol to yield each butanol (FB) fraction and the residual water fraction (WW). Finally, FD and FB fractions were assayed as is described below. A further separation scheme was performed with sample IBUN-02224. The crude extract from this cyanobacterial mat was fractionated over a DIOL cartridge (5 g) using the following mobile phase composition, Hex/EtOAc 8:2, Hex/EtOAc 1:1 EtOAc 100 %, EtOAc/MeOH 1:1, and MeOH and yielding five fractions of increasing polarity (FI-FV).

UHPLC-DAD-ELSD analysis of the FD and FB fractions

UHPLC analysis were performed in a Thermo Dionex ultimate 3000 system coupled to an ELSD Sedex 85 detector (Gain detector 10 and temperature 80 °C), using a C-8 column (Kinetex, 10 × 2.1, 1.7 μm), with a MeOH/H_2O gradient starting at 70 % (v/v) MeOH (for 3 min) reaching up to a 100 % (v/v) MeOH in 10 min. A 100 % MeOH was maintained for additional 10 min.

Cyanobacterial mat culture conditions

Small portions of cyanobacterial mats (1 g) were cleaned from debris and stored in sterile vials with seawater from the sampling site. Samples were kept under sunlight and brought to the laboratories of Universidad Nacional and Universidad Jorge Tadeo Lozano in Bogota. Once in the laboratory, samples were rinsed with sterile artificial seawater (salinity 35) and then suspended in sterile SWBG-11 culture medium (30 mL) [34]. Cyanobacterial samples were grown at 25 °C, with a 12 h light/dark regime and without aeration, seeking to not affect biofilm development. Cultured cyanobacteria were kept under these conditions for two months changing the SWBG-11 culture medium every other week.

Culture escalations were performed every two months. Surviving cyanobacterial samples were transferred to 100 mL in a 250-ml Erlenmeyer flask, maintaining the same culture conditions as described above. Then, a further escalation was done to 200 mL of SWBG-11 medium in 500-mL Erlenmeyer flasks, and two months afterwards they were transferred to 300 mL of SWBG-11 medium in 750-mL Erlenmeyer flasks.

Extraction and chemical partitions of cultured cyanobacteria

Cultured cyanobacterial samples were extracted using a DCM/MeOH (1:1) mixture as described previously, in the environmental sample extraction section. Solvents were removed under reduced pressure yielding a crude extract. Each crude extract was weighed, resuspended in water, and extracted with EtOAc.

Larvicidal bioassay against *Aedes aegypti*

Larvicidal assays were performed following Berry *et al*. [9]. *A. aegypti* eggs were allowed to hatch in hypoxic deionized water at 28 °C during 30-40 min. Assays were performed in 24-well plates. The tested fractions were either the FD or FB fractions from the environmental samples or the EtOAc fraction from the cultured cyanobacteria. All fractions were evaluated in triplicate. For the assay, fractions were resuspended in $CHCl_3$ and MeOH (1 mg/mL) to solubilize them; then 50 μg/mL of each test solubilized fraction were added in each well and mixed with deionized water to a final volume of 1 mL. Solvents were removed by evaporation before water was added to each well. Negative controls consisted of 10 μL of $CHCl_3$ and MeOH, the positive control consisted of 10 μg/mL 4,4'-DDT. Four freshly hatched (instar I) mosquito larvae were added to each well. Plates were incubated at 28 °C, with 12/12 h light/darkness periods for 6 days. Larval development was monitored during all four instars (six days). Each larva was fed with 20 μL of a 1 % liver powder solution. The number of dead/live larvae in each well was counted every 24 h. Test fractions that exhibited mortality greater than 50 % after 6 days were considered active.

Toxicity assessment against nauplii of the brine shrimp *Artemia salina*

Cysts of *A. salina* (1 g) were allowed to hatch in 1 L of artificial seawater (Salinity 38-40) at 28 °C with constant aeration and light. Assays were performed in 24-well plates. Tested fractions were either the FD or FB fractions from the environmental samples or the EtOAc fraction from the cultured cyanobacteria. All fractions were evaluated in triplicate. For the

assay, 10 μL or 50 μL from a 5 mg/mL solution of the FD and FB fractions or the EtOAc fraction of cultured cyanobacteria were added in each well and mixed with artificial sea water to reach a final volume of 2 mL. Acetone was used to solubilize the FD and FB fractions from environmental samples and the EtOAc fractions from cultured cyanobacteria. Negative controls consisted of 10 μL of acetone, the positive control consisted of 10 μg/mL of malathion. 10 *Artemia* nauplii were added to each well. Plates were incubated at 28 °C. After 24 h dead/live nauplii were counted in each well. Test fractions that exhibited a $LC_{50} \leq 12.5$ μg/mL after 24 hours were considered active [35].

Acetylcholinesterase inhibition bioassay

Crude extracts and fractions were tested for their AChE inhibitory activity following the Marston method [36]. Briefly, an AChE solution (4 UA/mL) was prepared in phosphate buffer 0.1 M, pH = 7.4, with BSA (1 mg/mL). 1-naphthyl acetate was used as a substrate solution and prepared in 96 % ethanol (2.5 mg/mL). A Fast-Blue Salt (FBS) dye solution in water (2.5 mg/mL) was prepared prior to use. All test fractions were resuspended in dichloromethane or methanol and applied on a TLC plate for the AChE inhibition test. Malathion (10 μg/spot) was used as a positive control. TLC plates were eluted with a mixture of n-hexane/EtOAc in a 7:3 ratio for FD fractions, whereas a mixture of $CHCl_3$/MeOH in a 9:1 ratio was used for the FB fractions of environmental samples. TLC plates were dried and sprayed uniformly with an AChE solution and incubated for 30 minutes at 37 °C. Plates were then sprayed with the 1-naphtyl-acetate solution and incubated for 30 min at 37 °C. Finally, the plates were sprayed with the FBS dye solution until a purple coloration was observed (1-2 minutes). The presence of white spots on the plate was associated with AChE inhibition [37].

Results and discussion

Cyanobacterial mat composition and culture

Thirty samples of cyanobacterial benthic mats were collected from different marine substrates such as sediments, algae, and soft corals. We have recently documented the potential allelopathic effects of cyanobacterial extracts from live corals and soft corals [38]. All collected cyanobacteria showed filamentous and turf morphologies (**Table 1**). The preliminary taxonomic classification based on morphometric parameters allowed the identification of cyanobacteria belonging to the genera *Phormidium*, *Symploca*, *Oscillatoria*, *Lyngbya*, *Pseudoanabaena*, *Leptolyngbya*, *Moorea*, and *Dapis*. A mat of Dapis

Table 1. Cyanobacterial mats from Colombian Caribbean Sea studied for their insecticidal potential. Collection data are included. **a)** Not registered mats at Instituto de Biotecnología de la Universidad Nacional de Colombia (IBUN) strain collection because they were not cultured. **b)** Identified as *Lyngbya* spp.-like. **c)** Sample identified using chemotaxonomic characters, unpublished results. **NI:** Non identified due to the quantity of collected biomass was not enough to do it, and the sample did not grow in culture conditions.

IBUN code	Mat sample	Taxonomic identification	Procedure	Collection year	Collection place
NR[a]	PNM-07	*Phormidium submembranaceum* / *Symploca hydnoides* consortium	Direct chemical study	2010	Providencia
NR[a]	PNM-08	*Phormidium submembranaceum* / *Symploca hydnoides* consortium	Direct chemical study	2010	Providencia
NR[a]	PNM-13	*Phormidium submembranaceum* / *Symploca hydnoides* consortium	Direct chemical study	2010	Providencia
NR[a]	PNM-18	*Phormidium submembranaceum* / *Symploca hydnoides* consortium	Direct chemical study	2009	Providencia
NR[a]	PNM-28	*Lyngbya* spp.[b]/ *Oscillatoria* spp.	Direct chemical study	2009	Providencia
IBUN-02213	PNM-C001	*Dapis pleousa* mat[c]	Direct chemical study and culture	2015	Providencia
IBUN-02214	PNM-C002	*Dapis pleousa* mat[c]	Direct chemical study and culture	2015	Providencia
IBUN-02215	PNM-C003	NI	Culture	2015	Providencia
IBUN-02216	PNM-C004	NI	Culture	2015	Providencia
IBUN-02220	PNM-C005	*Lyngbya* sp.[b] / *Pseudoanabaena* sp	Culture	2015	Providencia
IBUN-02221	PNM-C006	*Lyngbya* sp.[b] / *Phormidium* sp	Culture	2015	Providencia
IBUN-02222	PNM-C007	NI	Culture	2015	Providencia
IBUN-02223	PNM-C008	NI	Culture	2015	Providencia
IBUN-02224	PNM-C009	*Phormidium tenue*	Direct chemical study and culture	2015	Providencia
IBUN-02225	PNM-C010	*Phormidium* sp. mat	Direct chemical study and culture	2015	Providencia

IBUN-02226	PNM-C011	*Leptolyngbya* sp.	Culture	2015	Providencia
IBUN-02227	PNM-C012	NI	Culture	2015	Providencia
IBUN-02228	PNM-C013	NI	Culture	2015	Providencia
IBUN-02229	PNM-C014	NI	Culture	2015	Providencia
IBUN-02230	PNM-C015	NI	Culture	2015	Providencia
IBUN-02231	PNM-C016	*Phormidium* sp./ *Leptolyngbya* sp. mat	Culture	2015	Providencia
IBUN-02232	PNM-C017	NI	Culture	2015	Providencia
IBUN-02233	PNM-C018	NI	Culture	2015	Providencia
IBUN-02234	PNM-C019	NI	Culture	2015	Providencia
IBUN-03493	PNM-R001	*Phormidium* sp. mat	Direct chemical study	2016	Islas del Rosario
IBUN-03494	PNM-R002	*Phormidium* sp. mat	Direct chemical study	2016	Islas del Rosario
IBUN-03495	PNM-R003	*Moorea producens* mat	Direct chemical study and culture	2016	Islas del Rosario
IBUN-03496	PNM-R004	*Moorea producens* mat	Direct chemical study	2016	Islas del Rosario
IBUN-03497	PNM-R005	*Moorea producens* mat	Direct chemical study and culture	2016	Islas del Rosario
IBUN-03498	PNM-R006	*Moorea producens* mat	Direct chemical study	2016	Islas del Rosario

pleousa was identified using morphological and chemical traits, namely the production of malyngolide (unplublished data). Malyngolide is a diagnostic chemical marker for this species and may be used as a chemotaxonomical marker according to Engene *et al*. [39]. Some of the collected samples were not identified because of insufficient sampled biomass and failure to grow under laboratory conditions.

Among the collected cyanobacteria, *Lyngbya*-like mats were very conspicuous in the Caribbean Sea. Almost 350 compounds have been isolated from *Lyngbya*-like species, an unusual number for a single genus [34]. Results of morphological and molecular trait studies in *Lyngbya*, by Engene *et al*., revealed that this genus is a polyphyletic group. Consequently, the genus has been reassigned in three different genera, *Moorea* [16], *Okeania* [40], and *Dapis* [39]. However, there are still other *Lyngbya* species that have not been reclassified yet [34]. All these three genera produce a vast number of metabolites, many of which are genus or species-specific. These metabolites are used nowadays as chemotaxonomic markers [33].

Table 2. Bioassay results for FD, FB and DIOL fractions obtained from environmental mat samples. The *Aedes aegypti* larvae percent mortality was evaluated at 50 µg/ml. evaluation after six days, while the *Artemia salina* percent mortality was tested at 12.5 µg/ml after 24 hours. **a)** --: No tested. **b)** AChEI (AChE inhibitors): +++ high activity, + medium activity, - inactive (no inhibition observed).

Sample	Mat description	Fraction	Dry weight (mg)	Fraction weight (mg)	% *A. aegypti* larvae mortality	% *A. salina* mortality	AChEI[b]
Acetone control 2.5 %					--[a]	3	--[a]
$CHCl_3$ control (10 µl/well)					8	--[a]	-
MeOH control (10 µl/well)					0	--[a]	-
Malathion					--[a]	100 (10µg/ pozo)	+++ (10µg/ point)
DDT (10 µl/well)					100	--[a]	--[a]
PNM-07	*P. submembra--ceum / Symploca hydnoides* consortium	FD	54.9	429.8	17	**73**	++
		FB	54.9	438.5	17	**67**	+
PNM-08	*P. submembraceum / S. hydnoides* consortium	FD	175.8	183.6	8	**100**	++
		FB	175.8	63.3	14	**67**	++
PNM-13	*P. submembraceum / S. hydnoides* consortium	FD	150.0	423.7	8	**67**	++
		FB	150.0	255.4	9	**67**	+
PNM-18	*P. submembraceum / S. hydnoides* consortium	FD	16.0	249.8	**100**	37	+
		FB	16.0	107.3	25	17	+
PNM-28	*Lyngbya* spp. / *Oscillatoria* spp. consortium	FD	9.2	129.4	**90**	**53**	+++
		FB	9.2	110.6	0	**53**	-
IBUN-02213	*Dapis pleousa* mat	FD	725.5	2931.6	17	**67**	++
		FB	725.5	845.3	**58**	33	+

IBUN-02214	*Dapis pleousa* mat	FD	121.8	29.1	8	40	++
		FB	121.8	24.2	0	30	-
IBUN-02213	*Dapis pleousa* mat	FD	725.5	2931.6	17	**67**	++
		FB	725.5	845.3	**58**	33	+
IBUN-02214	*Dapis pleousa* mat	FD	121.8	29.1	8	40	++
		FB	121.8	24.2	0	30	-
IBUN-03493	*Phormidium* sp. mat	FD	28.3	191.2	**50**	**77**	++
		FB	28.3	124.1	0	37	-
IBUN-03494	*Phormidium* sp. mat	FD	23.2	141.4	**83**	**70**	++
		FB	23.2	50.8	17	30	-
IBUN-03495	*Moorea producens* mat	FD	247.7	285.2	**83**	**70**	++
		FB	247.7	104.9	0	23	+
IBUN-03496	*Moorea producens* mat	FD	315.2	1643.2	**100**	**100**	++
		FB	315.2	987.4	8,3	27	+
IBUN-03497	*Moorea producens* mat	FD	199.6	222.3	45	**100**	++
		FB	199.6	54.5	33	**83**	+
IBUN-03498	*Moorea producens* mat	FD	95.9	833.3	23	**100**	++
		FB	95.9	875.7	0	33	+
IBUN-0224	*Phormidium tenue*[d]	FI (Hex/EtOAc 8:2)	23.4	9.3	17	**71**	+
		FII (Hex/EtOAc 8:2)	23.4	12.5	8	**67**	+
		FIII (EtOAc 100%)	23.4	24.7	**58**	12	-
		FIV (EtOAc /MeOH 1:1)	23.4	126.2	0	9	-
		FV (MeOH 100%)	23.4	27.2	0	3	-

Some of the collected cyanobacterial mats were locally abundant, for instance *Dapis pleousa* (IBUN-02213) and *Moorea producens* (IBUN-03495, IBUN-03496, IBUN-03497, and IBUN-03498). Other mats were less common these included consortia of *Lyngbya* sp.-*Pseudoanabaena* sp. (IBUN-02220) and *Lyngbya* sp.-*Phormidium* sp. (IBUN-02221).

Cyanobacterial cultures and further escalation were initiated with the most common mats (Table 1 and Table 2). The least abundant mats were just cultured in order to obtain enough biomass for chemical studies. Thanks to these cyanobacteria cultures enough biomass was available to perform

thorough molecular characterizations of these microorganisms and to isolate, analyze, and elucidate their bioactive compounds. In a previous work, and following Bertin *et al*. [41], we established that the SWBG-11 culture medium was the most appropriate for culturing marine cyanobacteria (Unpublished data). Our formulation of the SWBG-11 medium (equivalent to natural eutrophication conditions [20]) included high concentrations of phosphorus, nitrogen, and iron (III).

The phases of acclimatization and establishment of cyanobacterial cultures were critical stages for the cyanobacteria cultures that required permanent monitoring. The survival of all environmental samples cultured (23 out of 23) is of the one most important achievements of this work (**Table 3**). In previous works survival rates of cultured environmental cyanobacteria rarely surpassed 50 % [19, 20, 42]. Our success can be attributed to effective control of cyanobacteria opportunistic populations, using various strategies such SWBG-11 medium replacement for oligotrophic medium (sterile seawater), manual rearing, antibiotic treatment (Cyclohexamide, 100 μg/mL), filament separation by ultrasonic treatment, centrifugation with glass beads, surfactant addition (e.g. sodium oleate), and subsequent serial dilutions [43]. One must keep in mind however, that the established cyanobacterial mats under laboratory conditions, were not necessarily the same consortia of the original environmental samples.

Another critical step in culturing cyanobacteria was escalating cultures to larger volumes. Although all environmental samples survived in small volumes of culture medium, only 17 of them survived the first escalation phase (final volume of 100 mL), 14 samples survived the second escalation phase (final volume of 200 mL), and 8 samples survived the third escalation phase (final volume of 300 mL). Some consortia may have been highly susceptible to mechanical stress during the escalation process (Table 3). Cyanobacteria that tolerated well the escalation process belonged to the genera *Lyngbya*, *Leptolyngbya*, and *Phormidium*. These are filamentous cyanobacteria with very narrow trichomes that may be effective for nutrient uptake [19, 20, 42]. After 9 months, only five samples attained enough biomass to provide enough material to obtain extracts and run larvicidal assays (Table 3).

Larvicide and ecotoxic activities of fractions from environmental cyanobacterial samples

As explained in the methods section, a crude extract from each sampled cyanobacterial mat with a wide polarity range was obtained with DCM/MeOH (1:1). Further fractionation yielded a fraction of low-medium

Table 3. Survival behavior of some cyanobacterial mats at laboratory conditions. **G:** growth at culture conditions after 4 months. **NG:** Non observable growth. **D:** death.

IBUN Code	Mat	Conditioning (30 mL)	Volume culture		
			100 mL	200 mL	300 mL
IBUN-02213	PNM-C001	G	G	G	NG
IBUN-02214	PNM-C002	G	G	G	NG
IBUN-02215	PNM-C003	G	G	G	NG
IBUN-02216	PNM-C004	G	G	G	NG
IBUN-02220	PNM-C005	G	G	G	G
IBUN-02221	PNM-C006	G	G	G	G
IBUN-02222	PNM-C007	G	G	G	NG
IBUN-02223	PNM-C008	G	G	G	NG
IBUN-02224	PNM-C009	G	G	G	G
IBUN-02225	PNM-C010	G	G	G	D
IBUN-02226	PNM-C011	G	G	G	G
IBUN-02227	PNM-C012	G	G	NG	NG
IBUN-02228	PNM-C013	G	D	NG	NG
IBUN-02229	PNM-C014	G	G	G	NG
NG	PNM-08	PNM-08	PNM-08	PNM-08	PNM-08
IBUN-02230	PNM-C015	G	D	NG	NG
IBUN-02231	PNM-C016	G	G	G	G
IBUN-02232	PNM-C017	G	G	D	NG
IBUN-02233	PNM-C018	G	G	D	NG
IBUN-02234	PNM-C019	G	G	G	NG
IBUN-03495	PNM-R003	G	NG	NG	NG
IBUN-03497	PNM-R005	G	NG	NG	NG

polarity (FD) and a fraction of medium-high polarity (FB). Aqueous layers, extracted with BuOH, were not tested due to their high salt content. All FD and FB fractions from the environmental samples, as well as the DIOL fractions (FI-FV) from sample IBUN-02224, were tested in the three bioassays. Results are summarized in Table 2.

Larvicidal activity tests against *A. aegypti* were monitored trough all four instars (six days in total). Larvae in the control solvent elicited a mortality below 10 %, which is within the range larval natural mortality [9]. Eight out of 35 fractions tested were active. Most of them corresponded to the FD fractions (with a low-medium polarity). These results are consistent with those reported by Harada [8] and Berry [9]. These studies detected larvicidal activity in the lipid fraction, due to the presence of unsaturated fatty acids (oleic, linoleic, and γ-linolenic acid), and polar fractions due to the presence of sulfated glycolipids [8, 9]. In our case, only a polar fraction, obtained from mat IBUN-02213 (*Depis pleousa*), showed larvicidal activity. Additionally, the FIII fraction (medium polarity) from *Phormidium tenue* (IBUN-2224) was active against *A. aegypti* larvae.

The FD fractions of the *Phormidium submembranaceum-Symploca hynoides* consortium (PNN-18) and *M. producens* (IBUN-03496) mats were the most active extracts against mosquito larvae (eliciting 100 % mortality at 50 μg/well). *Symploca* species are well known to produce potent, cytotoxic compounds such as symplostatin 1 and 2 [44, 45]. The *Moorea* genus is know as a rich source of bioactive natural compounds, however, there are no reports of insecticidal compounds isolated from this genus according to the Marinlit database [46]. The non-polar fraction (FD) of the *Lyngbya-Oscillatoria* consortium (PNM-28) revealed good larvicidal activity. Unsaturated fatty acids with larvicidal activity have been isolated from *Oscillatoria aghardii* [7]. This kind of compunds may be responsible for the activity observed in this fraction.

Both larvicidal activity and metabolic profiles of the *P. submembranaceum-S. hydnoides* consortia (samples PNM-07, PNM-08, PNM-13 and PNM-18) varied with collection year. Sample PNM-18, collected in 2009, showed great activity against mosquito larvae. However, samples PNM-07, PNM-08, and PNM-13, collected in 2010, were devoid of activity against *A. aegypti* larvae. This finding conforms to a previous metabolomic study revealing that the metabolic profiles of cyanobacterial mats collected in 2009 (e.g. PNM-18) differ from those of mats collected in 2010 (PNM-07, PNM-08, and PNM-13) [Unpublished data].

We also observed spatial variation in the bioactivity and metabolic profiles of FD fractions from *M. producens* mats collected at different locations. Samples IBUN-03495, IBUN-03496, IBUN-03497, and IBUN-03498) were collected off Isla Grande (Islas del Rosario) in June 2016 at different but nearby locations. The FD fractions from IBUN-03495 and IBUN-03496 elicited *A. aegypti* larvae mortalities of 83 % and 100 %, respectively, whereas samples IBUN-03497 and IBUN-03498 resulted in mortalities of 45 % and 23 %, respectively. The HPLC-ELSD analysis from these FD fractions showed different chemical profiles, revealing chemical profile differences between the most active and the least active fractions (**Fig. 2**). This suggests that differences in larvicidal activity may reflect varying metabolic profiles associated to particular microenvironmental conditions at each collecting site or ecological interactions taking place at the time of collection. A single cyanobacterial species may produce different metabolites depending on biotic and abiotic conditions such as temperature, light intensity, or nutrient availability [47].

Studies taking into consideration both the variation of environmental parameters and ecological interactions (i.e. space competition or protection from grazing), must be conducted before ascribing metabolite production to a particular factor. On the other hand, the observed metabolic variation could be explained by genetic differences. For instance, the *Phormidium* sp.

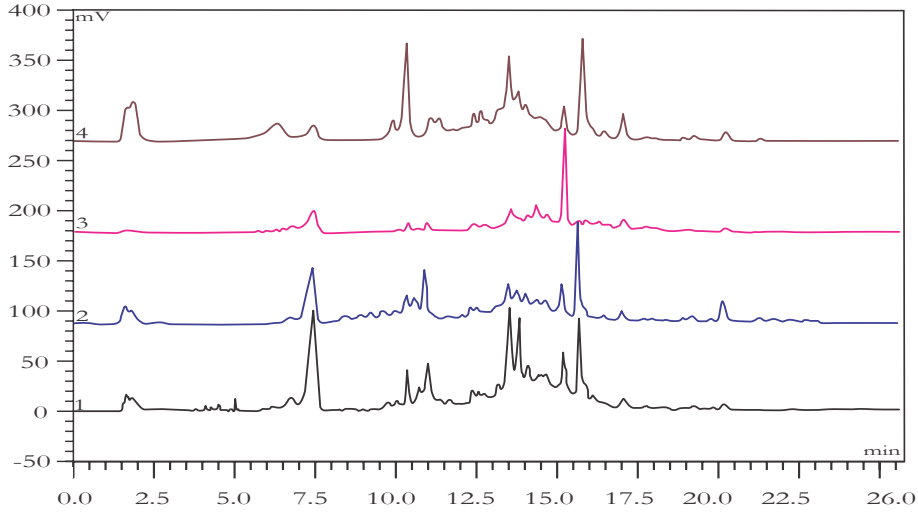

Figure 2. HPLC-ELSD chromatograms (normalized) of four *Moorea producens* FD fractions: IBUN-03495 (active, black line), IBUN-03496 (active, blue line), IBUN-03497 (inactive, pink line), and IBUN-03498 (inactive, brown line). The four *M. producens* mats were collected at different locations and exhibited different biological activities as well as different chemical profiles.

samples IBUN-03493 and IBUN-03494) were both collected in Providencia in June 2016. Both samples, showed differences in larvicidal activity at 50 μg/mL (eliciting 0 % and 83 % larvae mortality, respectively), but their chromatograms were very similar (**Fig. 3**). Possibly, the compounds responsible for larvicidal activity were in very low quantities and were not readily detected in the chemical profiles.

Lethality tests against the brine shrimp *A. salina* are a general approach to evaluate the toxicity of extracts, fractions, or compounds. However, some researchers have correlated those results with insecticidal activity [48] and to the selectivity of a particular compound acting as an insecticide [49]. We expected a promising larvicidal substance to be very active against *A. aegypti* larvae and exhibiting little toxicity against *A. salina.* However, we did not discard extracts active against both *A. aegypti* and *A. salina*. In our toxicity assays, test fractions that exhibited a $LC_{50} \leq 12.5$ μg/mL after 24 hours were considered active. All the non-polar (FD) fractions caused some mortality, but only 12 out of 15 were considered toxic, while only 5 of the 15 polar fractions tested (FB) were toxic against nauplii of *A. salina*.

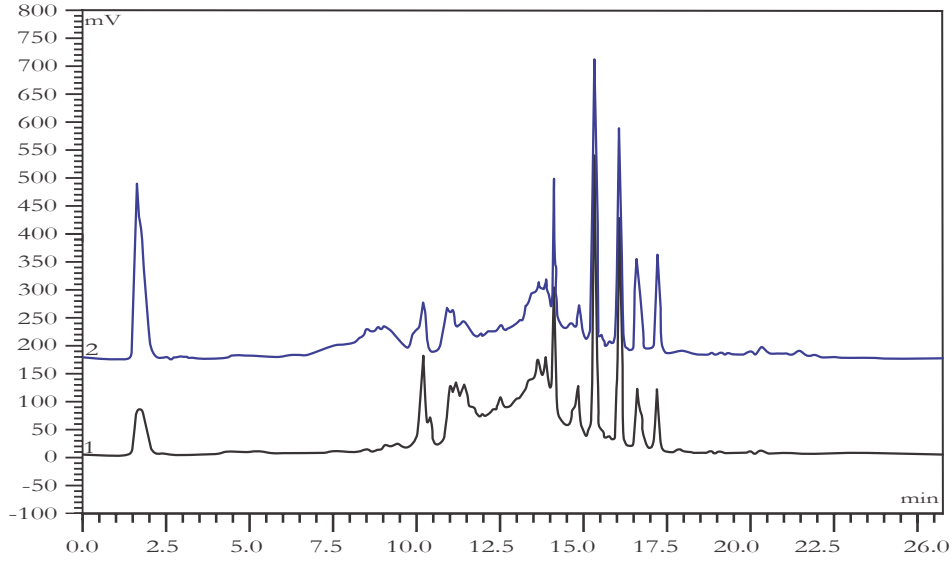

Figure 3. HPLC-ELSD chromatograms (normalized) of two *Phormidium* sp FD fractions: IBUN-03493 (black line) and IBUN-03494 (blue line). These two FD fractions exhibited different biological activities but had similar chemical profiles.

All FD fractions were rich in lipidic compounds. Some fatty acids have been reported active against *A. salina* and other arthropods, inhibiting Na^+ and K^+ ATPases [50]. The presence of fatty acids in FD fractions could explain the lethality results against *A. salina*. The most toxic extracts were obtained from samples of *M. producens* (IBUN-03495, IBUN-0349, IBUN-03497, and IBUN-03498) and mixed consortia of *P. submembranaceum-S. hynoides* showed varying toxicity. Three out of the four consortia extracts were toxic. For instance, sample PNM-08 showed a 100 % mortality at a concentration of 50 μg/mL, whereas extract PNM-18 was not toxic at any of the concentrations tested. Again, a varying degree in biological activity became evident in samples collected in different years. Samples PNM-07, PNM-08, and PNM-13, collected in 2010 at Isla Grande, were toxic to Artemia nauplii. However, a sample of *M. producens* collected at the same site in 2009 did not exhibit such activity. Discussing the putative causes of such variation is out of the scope of this paper, but they are likely related to the interaction of environmental variables such as increased nutrient input and increased water temperatures affecting the reefs at Islas del Rosario [51]. Our results failed to establish a positive correlation between larvicidal activity and toxicity to *A. salina*, as opposed to other reports in the literature [48].

AChE inhibitory effect of fractions from environmental cyanobacterial samples

In the AChE inhibition assay, all the FD fractions tested inhibited acetylcholinesterase. The most active samples consisted of *Lyngbya-Oscillatoria* consortia (sample PNM-28); revealing strong activity against *A. aegypti* larvae and low toxicity against *A. salina*. This result could reflect insecticidal activity mediated by AChE inhibition. Interestingly, some of the health hazards linked to freshwater cyanobacterial blooms have been related to acetylcholinesterase inhibition [52]. Furthermore, neurotoxic effects on birds are caused by cyanobacterial toxins able to inhibit cholinesterases [53]. The most representative AChE inhibitor compound from cyanobacterial sources is anatoxin-a, isolated from the planktonic species *Anabaena flos-aquae* and produced by other freshwater cyanobacteria [54].

Before conducting the AChE inhibition assays, we hypothesized that strong AChE inhibitors would show significant larvicidal activity against *A. aegypti*, following the mode of action of insecticidal organophosphates and carbamates [55]. However, some of the cyanobacterial samples tested showed strong AChE inhibition without being larvicidal. This could be partly ascribed to structural differences in the acetylcholinesterase of insects compared to that

of the model organism from which the test enzyme was obtained (*i.e.* the fish *Electrophorus electricus*). Alternatively, we propose that compounds in the test extracts may not have crossed insect biological barriers, as an explanation for the inhibition activity on the enzyme and the absence of activity on the mosquito larvae. Nevertheless, this hypothesis remains to the be tested.

Larvicidal extracts that do not inhibit acetylcholinesterase are promising insecticides because of the wide-spread insecticide resistance against this target [56, 57]. Additionally, a mechanism of action that does not involve this enzymatic target, allows for better selectivity and less toxicity in non-target organisms. This pattern was observed in the FD fraction of sample PNM-18 (from a *P. submembraceum-S. hydnoides* consortium) as well as in fraction III of the extract from *D. pleousa* (sample IBUN-02214).

In summary, the most promising cyanobacterial extracts were obtained from *M. producens* (samples IBUN-03495 and IBUN-03496), followed by extracts from *P. submembranaceum-S. hynoides* mixed consortium (PNM-18), *Lyngbya* spp.-*Oscillatoria* spp. mixed consortium (PNM-28), and *Phormidium* sp. (IBUN-03494). Mats of *M. producens* were locally abundant, thick, and conspicuous but exhibited large chemical variation depending on collection sites and dates. Extracts from mixed cyanobacterial consortia and from *Phormidium* sp. were less abundant and yielded small amounts of crude extracts. The larvicidal activity of the FIII fraction of the Phormidium tenue extract (IBUN-0224) showed larvicidal activity being moderately toxic against *A. salina*. Hence, this extract could be considered a selective larvicidal agent. Additionally, this fraction did not inhibit AChE, suggesting a different mode of action, which could be of interest in the light of the recurrent reports of insecticide resistance.

Larvicide and ecotoxic activities of cultured cyanobacteria extracts

Crude extracts of five cyanobacterial cultures were partitioned between EtOAc and water to yield a fraction of low-medium polarity. Three out of the five fractions tested, from cultures of the genera *Phormidium* and *Lyngbya*, showed strong larvicidal activity (100 % mortality; **Table 4**), and two fractions from cultures the genus *Leptolyngbya* exhibited mild-to-low larvicidal activity ($\leq$ 40 % mortality; Table 4). In contrast to *Phormidium* fractions, those from *Leptolyngbya* were less active against mosquito larvae and were characterized by their fast growth. It is thought that fast growing cyanobacteria are more effective in nutrient uptake, whereas slow growing mats invest more energy resources in defensive compound biosynthesis. Studies from *Leptolyngbya* collected in Florida showed larvicidal activity, but also spatial and temporal metabolic variations with direct consequences on their biological activity [7, 9].

Table 4. Bioassays results for crude extract of cultured cyanobacteria. The *Aedes aegypti* larvae percent mortality was evaluated at 50 µg/ml. evaluation, while the *Artemia salina* percent mortality was tested at 12.5 µg/ml after six days.

IBUN code	Mat sample	Taxonomic identification	% *A. aegypti* larvae Mortality	% *A. salina* mortality	AChEIb
IBUN-02220	PNM-C005	*Lyngbya* sp./ *Pseudoanabaena* sp	100	56	-
IBUN-02221	PNM-C006	*Lyngbya* sp./ *Phormidium* sp	100	63	-
IBUN-02224	PNM-C009	*Phormidium* tenueb	97	11	++
IBUN-02226	PNM-C011	*Leptolyngbya* sp.	40	6	++
IBUN-02231	PNM-C016	*Phormidium* sp./ *Leptolyngbya* sp. mat	19	60	+
	Malathion		100	100	++
	Negative control		r	2	-

Larvicidal extracts from *Lyngbya* consortia (samples IBUN-02220 and IBUN-02221) were also active against *A. salina* nauplii, showing activity comparable to that of organophosphate insecticides [58]. They did not inhibit the acetylcholinesterase, however. The organic extract of cultured *Phormidium tenue* (sample IBUN-02224) caused a 97 % mortality of *A. aegypti* larvae after six days of exposure, had a moderate AChE inhibitory activity, and exhibited very low toxicity against *A. salina* nauplii (Table 4). After the assay, surviving mosquito larvae stopped their development in instars 2 and 3, suggesting an ontogenic development interruption elicited by this extract. There are few reports on this regard because most studies evaluate mosquito mortality in the third and fourth instars of their development. Growth interruption implies that mosquito larvae will not reach their adult stage, and therefore, they will not become vectors of viral diseases.

Conclusions

The mats of marine benthic cyanobacteria subject of this study were complex consortia belonging to the genera *Phormidium*, *Symploca*, *Oscillatoria*, *Lyngbya*, *Pseudoanabaena*, *Leptolyngbya*, *Moorea*, and *Dapis* in varying proportions. We were able to culture and perform gradual escalations of

these cyanobacteria cultures. The survival rate of our cultured environmental samples can be attributed to our reformulation of the SWBG-11 medium, by enriching it with high concentrations of phosphorus, nitrogen, and iron (III). Notwithstanding, only a few cultured filamentous cyanobacteria belonging to the genera *Lyngbya*, *Leptolyngbya*, and *Phormidium* provided enough biomass for bioassays.

In general, the non-polar fractions of the tested cyanobacterial extracts showed the greatest larvicidal activity, were toxic against A. salina nauplii, and showed significant AChE inhibition. Nonetheless, the polar fraction from *D. pleuosa* showed larvicidal activity without being toxic to *A. salina* nauplii. Since benthic mats of this cyanobacterium can be locally abundant, they are worth a thorough chemical study. The non-polar fractions from the extract of *M. producens* (IBUN-03496) showed strong larvicidal activity being toxic to *A. salina* nauplii. The medium-polarity fraction from *Phormidium tenue* showed interesting larvicidal activity, without being toxic against *A. salina* nauplii, and without inhibiting AChE.

We found important differences in *A. egypti* larvicidal activity and metabolic profiles of cyanobacterial samples collected at the same sampling sites in different years. For example, between mixed consortia of *Phormidium submembranaceum-Symploca hynoides* collected in Old Providence in 2009 and 2010. From the cultured cyanobacteria mats, the extracts of *Phormidium* and *Lyngbya* showed strong larvicidal activity but only the extract of *Phormidium tenue* showed larvicidal activity with a potential low toxicity, as determined in the lethality assay against *A. salina* nauplii.

Despite their chemical profile variations, our marine benthic cyanobacteria extracts could be further developed as control mechanisms against larval stages of *A. aegypti* due to their larval toxicity, potential selective mechanisms, and low ecotoxicity in many cases. The cultured of marine benthic cyanobacteria requires further explored to provide enough biomass and to obtain interesting bioactive compounds.

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Conflict of interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject or materials discussed in this manuscript.

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Cianobacterias ambientales y cultivadas como fuentes de larvicidas de *Aedes aegypti*

Resumen: En países tropicales, el control del mosquito *Aedes aegypti* es una prioridad de salud pública a causa de su papel como vector de importantes enfermedades virales. Las cianobacterias marinas son una reconocida fuente de compuestos bioactivos y constituyen una fuente potencial de insecticidas útiles para el control de las poblaciones de mosquitos y la prevención de brotes epidémicos. En el presente trabajo, 30 colonias de cianobacterias bentónicas pertenecientes a los géneros *Phormidium*, *Symploca*, *Oscillatoria*, *Lyngbya*, *Pseudoanabaena*, *Leptolyngbya*, *Moorea* y *Dapis* fueron recolectadas en la isla de Providencia y las Islas del Rosario (en el Caribe colombiano). Las colonias de las cuales se tenía suficiente biomasa (muestras ambientales) fueron sometidas a extracción química y obtención de fracciones orgánicas. Las colonias muestreadas con biomasa insuficiente fueron primero cultivadas en el laboratorio. En tres bioensayos se evaluaron las fracciones orgánicas de las muestras ambientales para determinar: (i) actividad larvicida contra *A. aegypti*, (ii) ecotoxocidad: toxicidad contra los nauplios de artemia (*Artemia salina*) y (iii) inhibición de la acetilcolinesterasa. Las fracciones polares de un extracto de *Dapis pleuosa* mostraron actividad larvicida sin ser tóxicas para los nauplios de *A. salina*. Los extractos de *Moorea producens* mostraron la mayor toxicidad contra las larvas de *A. aegypti* y los nauplios de *A. salina*. De las 23 muestras de cianobacterias cultivadas, únicamente cinco crecieron en condiciones de laboratorio y produjeron suficiente biomasa para obtener de ellas extractos orgánicos. De estas, tres extractos mostraron actividad larvicida fuerte, pero solo el extracto de *Phormidium tenue* mostró toxicidad reducida contra los nauplios de *A. salina*. Dependiendo de los sitios y las fechas de colección, se detectó variación entre los perfiles químicos de los consorcios de cianobacterias y su actividad larvicida. Estos hallazgos sugieren que, a pesar de la variación de estos perfiles, los extractos de cianobacterias marinas pueden desarrollarse como agentes de control efectivo contra vectores de insectos en sus estados larvales. El cultivo de cianobacterias bénticas marinas debe ser explorado con más detalle para proporcionar biomasa suficiente que permita la identificación de compuestos bioactivos con aplicaciones en salud pública.

Palabras clave: Cianobacterias; *Aedes aegypti*; *Artemia salina*; actividad larvicida; inhibidores de la acetilcolinesterasa; cultivos de cianobacterias.

Cianobactérias ambientais e cultivadas como fonte de larvicidas de *Aedes aegypti*

Resumo: Em países tropicais, o controle do mosquito *Aedes aegypti* é uma prioridade de saúde pública devido a seu papel como vetor de importantes doenças virais. As cianobactérias marinhas são uma fonte conhecida de compostos bioativos e constituem uma potencial fonte de inseticidas úteis para o controle da população das populações de mosquito e prevenção de surtos epidêmicos. No presente trabalho, 30 colônias de cianobactérias bentônicas pertencentes aos gêneros *Phormidium*, *Symploca*, *Oscillatoria*, *Lyngbya*, *Pseudoanabaena*, *Leptolyngbya*, *Moorea* e *Dapis* foram coletadas nas ilhas de Providencia e Islas de Rosario (Caribe Colombiano). As amostras de colônias com suficiente biomassa (amostras ambientais) foram submetidas a extração química e obtenção de frações orgânicas. As amostras de colônias com biomassa insuficiente foram primeiro cultivadas em laboratório. As frações orgânicas das amostras ambientais foram avaliadas em três bioensaios para determinar: (i) atividade larvicida contra *A. aegypti*, (ii) ecotoxicidade, toxicidade contra os náuplios de artemia (*Artemia salina*) e (iii) inibição da acetilcolinesterase. As frações polares de um extrato de *Dapis pleuosa* mostraram atividade larvicida sem serem toxicas para os náuplios de *A. salina*. Os extratos de *Moorea producens* mostraram a maior toxicidade contra as larvas de *A. aegypti* e contra os náuplios de *A. salina*. Das 23 amostras de cianobactérias cultivadas, unicamente cinco cresceram em condições de laboratório, produzindo suficiente biomassa para a obtenção de extratos orgânicos. De estas, três extratos mostraram atividade larvicida forte, mas somente o extrato Phormidium tenue de mostrou toxicidade reduzida contra os náuplios de *A. salina*. Detectou-se variação entre os perfis químicos dos consórcios das cianobactérias e sua atividade larvicida, dependendo dos locais e datas de coleta. Estes resultados sugerem que, apesar da variação de estes perfis químicos, os extratos das cianobactérias bentônicas marinhas podem ser desenvolvidos como agentes de controle efetivo contra vetores de insetos em seus estágios de larva. A cultura de cianobactérias bentônicas marinhas deve ser explorada com mais detalhes para proporcionar biomassa suficiente que permita a identificação de compostos bioativos com aplicações em saúde pública.

Palavras-chave: Cianobactérias; *Aedes aegypti*; *Artemia salina*; atividade larvicida; inibidores de acetilcolinesterase; cultura de cianobactérias.

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