# Environmental Homeopathy: Homeopathic Potencies Regulate the Toxicity and Growth of *Raphidiopsis raciborskii* (Cyanobacteria) and can be Tracked Physico-Chemically. Part 1: Biological Results

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## Abstract

**Introduction** Cyanobacteria are microorganisms found in many parts of the world and several genera, such as *Raphidiopsis raciborskii*, are producers of cyanotoxins. Homeopathic potencies have been found to modulate toxicity in different biological models, and the present study endeavors to discover whether this might also be the case with cyanobacteria.

**Objectives** Our objective was to investigate the possible effects of homeopathic potencies on the resilience of *Artemia franciscana* (brine shrimp) embryos to saxitoxin (STX; cyanotoxin) and on controlling the growth of *R. raciborskii in vitro*.

**Method** *A. franciscana* cysts were cultivated in seawater in 96-well plates to evaluate the hatching rate and vitality, plus the gene expression of heat shock proteins (HSPs), after being challenged with *R. raciborskii* extract containing 2.5 µg/L of STX and treated with different homeopathic potencies. Untreated wells were used as controls ("baseline"). Potencies were chosen from a screening process based on seven selected homeopathic preparations according to the similitude of STX symptoms (*Sulphur*, *Zincum metallicum*, *Nitric acidum*, *Plumbum metallicum*, *Mercurius solubilis*, *Phosphoric acidum*, Isotherapic from *R. raciborskii* extract; all at 6cH, 30cH and 200cH). Cultures of *R. raciborskii* maintained in an artificial seawater medium were equally treated with screened homeopathic potencies selected from the same list but specifically for their growth control as a function of time.

**Results** A 15% lower rate of hatching of *A. franciscana* cysts was observed after treatment with *Nitric acidum* 6cH in comparison with baseline ($p = 0.05$). A complete toxicity reversal was seen after treatment with Isotherapic 200cH, with a 23-fold increase of *Hsp 26* gene expression ($p = 0.023$) and a 24-fold increase of *p26* gene

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expression ($p \leq 0.001$) in relation to baseline. *Nitric acidum* 200cH and *Mercurius solubilis* 30cH limited the exponential growth of cyanobacteria up to 95% and 85% respectively ($p \leq 0.003$) in relation to baseline. Succussed water presented only a transitory 50% inhibition effect.

**Conclusion** Isotherapic 200cH improved *A. franciscana* bioresilience to STX; *Nitric acidum* 200cH and *Mercurius solubilis* 30cH showed the optimal performance on limiting *R. raciborskii* growth. The results point to the potential of homeopathic potencies to mitigate environmental problems related to water quality.

## Introduction

Cyanobacteria are photosynthetic prokaryotic microorganisms that colonize all ecosystems on the planet. These organisms can form phytoplankton blooms in bodies of water. Some genera of cyanobacteria can produce cyanotoxins regardless of morphology and cell presentation (colonial or filamentous).

*Raphidiopsis raciborskii* (formerly termed *Cylindrospermopsis raciborskii*) is a cyanobacterium that produces neurotoxins named saxitoxins. It multiplies in freshwater rich in metals and organic matter, a clear indicator of water pollution. These bacteria form blooms in large water reservoirs and springs.[1–4]

Saxitoxin (STX) is an alkaloid containing seven nitrogen atoms in its structure. *R. raciborskii* produces it and, when ingested in drinking water, can block sodium and calcium channels, inhibiting nerve conduction and affecting potassium permeability, which leads to neuro-muscular blocking, diaphragm paralysis and death. In extreme cases, symptoms can begin 5 minutes after ingestion and death can occur within 2 to 12 hours. In non-lethal doses, the signs and symptoms are transient and disappear within 1 to 6 days.[5] In mice, microcrustaceans (*Daphnia similis*) and water fleas (*Ceriodaphnia dubia*), the effects of *R. raciborskii* extracts can be recognized by signs of neurotoxicity, such as tremors, convulsions (in mice) and immobilization.[6] Vilar & Molica (2020) observed changes in *R. raciborskii* growth and the production of saxitoxins after changes in water pH and dissolved carbon content.[7] The chronic effects of STX intoxication have still not been clearly elucidated. However, two significant consequences have been recently documented: adverse effects on aversive memory and delayed recovery from mammalian muscle injuries.[5]

The presence of toxic cyanobacterial populations in reservoir waters implies potential damage to human and animal health since the sources are used for various purposes such as agriculture, aquaculture, fishing, home use and leisure. In addition to ingesting cyanotoxins, another harmful contamination route is the consumption of aquatic organisms since cyanotoxins can accumulate in these organisms' muscles.[8,9]

The microcrustacean *Artemia* spp. (brine shrimp) is commonly used as an experimental model for toxicity tests.[10] A particular characteristic of the genus *Artemia* is the ability to keep embryos in diapause (a quiescent stage) in the presence of hostile conditions in the aquatic environment. In this way, the basal metabolism of the embryos remains reduced, and there is greater tolerance to any stressful factor from the external environment, such as toxins, temperature variations, dissection and others. Although the production of heat shock proteins (HSPs) is a crucial factor in this process,[11–15] the structure of the cyst is also quite peculiar, composed of a vitrified shell of non-glucose-reducing disaccharides, such as trehalose, which form bridges of hydrogen with phospholipids and macromolecules, giving the cysts excellent resistance. Trehalose also serves as an energy source for the embryo at diapause termination.[16,17]

Some studies have used *Artemia franciscana*[18] for toxicological tests involving cyanobacteria. A study by Sirvec et al. in 2016 sought to understand the massive death rate of several fish species in Lake Aleksandrovac, Serbia, which occurred during the flowering of *R. raciborskii*. Since high concentrations of chlorophyll a and pheophytin occurred in the lake, samples of phytoplankton bloom were harvested and tested in laboratory conditions using *A. franciscana* specimens kept in seawater: thus, the toxic compounds could be identified.[18] Natural extracts from a bloom of marine cyanobacterium *Trichodesmium*, another STX producer, proved to be lethal in bioassays with the genus Artemia. Thus, *Artemia* spp., a potential bioaccumulator in the marine food chain, can be considered a good model for identifying cyanotoxins.[19,20] Also, *Artemia spp.* are deemed to be non-sentient organisms, which significantly facilitates the ethical aspects involving their use in research.[10,21]

Recently, we have observed bioresilience processes in a model of *Artemia salina* cyst hatching by inserting isotherapic potencies of toxic agents into the culture water,[22–24] being prepared according to the official homeopathic pharmacopeia.[25] In Pinto et al, 2021, treating 20,000 cysts exposed to mercury chloride at 10% lethal concentration with the respective isotherapic prolonged the diapause period, protecting the embryos from direct contact with the toxic substance up to its evaporation.[22] Similar studies using high potencies of homeopathic arsenic performed in plants and microorganisms have shown similar effects.[26–28]

Given that such homeopathic dilutions often exceed the limit of Avogadro's number, it is necessary to consider the possible existence of mechanisms other than purely biochemical ones to explain the observed protective effects described above. From a biological point of view, what



appears to be an increase in adaptive processes (or "hormesis") has been frequently observed in living systems treated with homeopathic dilutions under a range of different circumstances.[29–31] Furthermore, such biological effects have shown close correspondence with physicochemical changes in previous microcrustacean studies.[22] These physicochemical changes in water can be monitored through interactions with solvatochromic dyes[24,32] based on the method developed by Cartwright.[32–38] Correspondence between biological effects and physicochemical changes has also been observed in other situations, in both laboratory[39] and natural[35] conditions.

The present study is justified by the need to look for cheap and effective technologies that focus on a familiar worldwide environmental problem: excessive cyanobacterial growth in water sources. In this Part 1 of a two-part report, the effects observed in biological models are described. Part 2 describes the physicochemical features of the most effective potencies and the corresponding treated media (seawater and artificial seawater medium [ASM]-1): it focuses on their interaction with solvatochromic dyes and variations in pH, conductivity and temperature.[40]

## Materials and Methods

The study design was organized as follows:

(1) Evaluation of *R. raciborskii* toxicity on *A. franciscana* and observation of which homeopathic potencies could mitigate it through cyst-hatching bioresilience and gene expression of heat-shock proteins.
(2) Evaluation of *R. raciborskii* growth rate after treatment with homeopathic potencies poured into the culture medium.

The homeopathic potencies were chosen from a standard screening process in both cases. The study design details are shown in **Diagram 1**.

### Toxicity of *Raphidiopsis raciborskii* extract on *Artemia franciscana* Cyst Hatching

The extracts and samples of *R. raciborskii* were provided by the Laboratory of Cyanobacteria and Phycotoxins, Federal University of Rio Grande (FURG), Brazil, for conducting the experiments at the Research Center of University Paulista (UNIP), São Paulo, Brazil. The standard strain of *R. raciborskii*—labeled the T3 strain—was originally isolated from the Taquacetuba arm of the Billings Reservoir in São Paulo, Brazil. The raw extracts from different batches were previously prepared in hydrochloric acid 0.05M, as described by Lagos et al.[2]

The toxicity of different batches of T3 extract was evaluated in a preliminary test on the hatching rate of *A. franciscana* cyst (Maramar-pet, Arraial do Cabo, Brazil) at different times (24, 48 and 72 hours). Next, a second assay was performed to test different concentrations of the chosen batch on nauplii viability to determine the ideal toxicity level for the subsequent experiments.

Environmental temperature and humidity were also monitored using a pre-calibrated thermo-hygrometer (JIAXI, Shanghai, China), certified on February 10, 2022. The unhatched cysts and born nauplii (larvae) were observed in each well using a digital high-resolution magnifying pen-type microscope—1,000x zoom camera, 2.0 megapixels, USB, 6 LEDs (Digital Microscope, Beijing, China), coupled to a computer (Yoga 520, Lenovo, Brazil), as previously described.[22–24]

In the first preliminary assay, *A. franciscana* cysts were distributed in 96-well microplates from an aqueous suspension containing 35 mg of cyst in 200 mL of artificial seawater or 3% marine salt solution (Red Sea Aquatics, London, UK). This was sufficient to obtain $28 \pm 9$ cysts for each fraction of 100 µL to be inserted into each well. Rows of 5 wells were completed for 250 µL of seawater containing 1% T3 extract from each batch. Untreated wells ("baseline") and 1% hydrochloric acid 0.05M were used as controls. This proportion was chosen after a pilot study to identify the hydrochloric acid concentration presenting no significant toxicity on cysts since it is used as a vehicle of T3 extracts. The sum of cysts and nauplii obtained from each row of wells and the respective hatching rate was obtained to compose each time point. The results were presented in a descriptive semi-quantitative table. The total number of evaluated cysts was 1,246 at this preliminary step. A descriptive semi-quantitative statistical analysis was used.

Samples of different batches of the acid extract of *R. raciborskii* were placed in each well, named X, Y, Z, 5, 6, 7, 8, according to their STX equivalent concentration, as previously calculated by comparison to a set of SXT standards variants purchased from the National Research Council of Canada, and analyzed by high-performance liquid chromatography with fluorescence detection methods, as described by others.[41,42] The known amount of STX per liter of each extract is described in ►**Supplementary file 1** (available in the online version).

In the second assay, extract 5 was chosen to evaluate the toxicity on nauplii survival, according to the results obtained in the first one. Rows of 8 wells containing $8 \pm 3$ cysts were completed for 250 µL of seawater containing T3 extract in different concentrations. Untreated wells (baseline) and 1% hydrochloric acid 0.05M were used as controls. After 48 hours of challenge, the sum of live and dead nauplii obtained from each row of wells and the respective ratio were used to compose each data point. The results were presented in a descriptive semi-quantitative table. The total number of evaluated cysts was 521 to identify the best concentration. A descriptive semi-quantitative statistical analysis was used.

To avoid eventual interference of electromagnetic environmental fluctuations due to the known sensitivity of this species,[22,23] all assays involving *A. franciscana* were performed in a Faraday cage with 300 micro-LED bulbs inside (►**Supplementary file 2**, available in the online version), allowing a constant low magnetic flow (0.06 µT at 50 Hz; Smart-Sensor Intel Instruments, AS 1392, Singapore) that is near the local terrestrial field measured outside the cage,[31] and crucial for the homeopathic potency activity,[43] with enough light to induce cysts hatching during incubation.

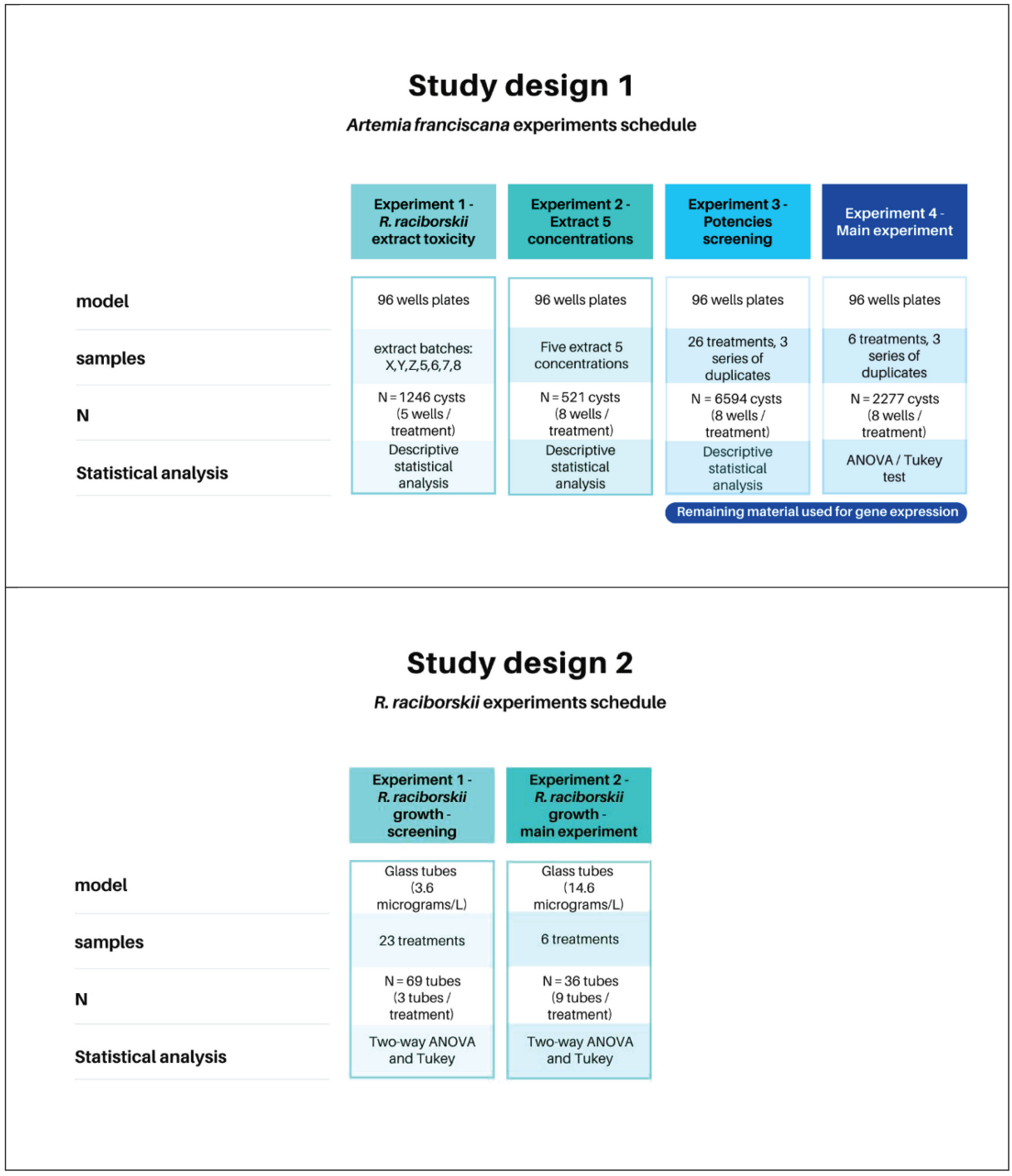



**Diagram 1** Diagram of the study design. (1) *Artemia franciscana* experiments; (2) *R. raciborskii* experiments.

Considering previous studies, the same lunar cycle phase was standardized in all experimental series,[22,23] now establishing the first-quarter moon as the standard timepoint.

### Screening of Homeopathic Potencies for Protection of *Artemia franciscana* from Toxicity of *Raphidiopsis raciborskii* Extract

Besides the isotherapeutics (homeopathic dilutions prepared from the *R. racibosrskii* extract 5), additional homeopathic preparations were chosen to be tested in a preliminary screening assay. The choice was based on the symptoms caused by STX in humans and the main biological cyanobacteria features (similitude). A group of three homeopath veterinarians and one homeopath physician derived the list of medicines by consensus. The criteria for composing the list were based on (1) cyanobacterial biological features (using dissolved metals to improve their growth, generating bad-smelling/tasting sensations, and excessive growth with destructive effects on other species living in the same environment)[1–4]; and (2) clinical-toxicological features of STXs in humans (dizziness, numbness in the mouth and extremities of the body, tachycardia, muscle weakness,

memory debility, nausea and vomiting, hyperemic skin lesions, eye irritation, nasal obstruction and conjunctivitis).[5]

Three potency levels were evaluated for each homeopathic medicine—6cH, 30cH and 200cH—according to protocols used in previous studies on brine shrimps.[22–24]

Thus, in this first screening study, the medicines tested were:

- *Sulphur* (an important element involved in cyanobacteria growth[4]).
- *Zincum metallicum* (a trace metal involved in cyanobacteria growth[9]; proving symptoms: extremities, weakness and tremors, conjunctivitis[44]).
- *Nitric acidum* (nitrogen is a crucial element for cyanobacteria growth[9] and the main chemical element in the STX molecule[5]; the behavior of cyanobacteria when growing excessively is very destructive concerning the other species that live in the same ecosystem[1–3,5,44]).
- *Plumbum metallicum* (lead is a trace metal involved in cyanobacteria growth control[9]; proving symptoms: extremities, weakness and tremors; paresis[44]).
- *Mercurius solubilis* (proving symptoms: local inflammation of skin and mucosae; mouth neuralgia; bad-smelling perspiration; paralysis of the extremities; nausea; vomiting;[44] all of them also present in STX intoxication[5]).
- *Phosphoric acidum* (phosphorus is another crucial element for cyanobacteria growth[9]; proving symptoms: muscle debility, dizziness[44])
- Isotherapeutics prepared from *R. raciborskii* extract 5.

The controls were:

- Unchallenged, non-treated, cysts (baseline).
- Hydrochloric acid 1cH ($5 \times 10^{-4}$ M).
- Autoclaved pure water.
- Succussed autoclaved pure water.

Stock potencies were prepared in an ANVISA (National Agency for Sanitary Surveillance) registered homeopathic pharmacy in São Paulo. They were supplied at one potency level below the working dilution, 5cH, 29cH and 199cH, using 10% alcohol as a solvent. The working potencies were prepared 1 day before the experiments, being the last 1:100 dilution made in autoclaved purified water (SmartPak Direct Q3 with Biopak filters—Merck–Millipore, Darmstadt, Germany), and succussed automatically in a robotic mechanical arm (Denise-Autic, São Paulo, Brazil) to shake the glass flasks 100 times vertically before it stops. After this process, all flasks were randomly coded by someone not involved with the experiment, and the codes remained closed in an envelope up to the final statistical analysis. Thus, the whole experimental procedure was blinded.

The cyst cultures were made in microplates, as described above. Before use, the working potencies were filtered in a 0.22 µm mesh filter (Merck–Millipore, Darmstadt, Germany). The proportion between treatment and seawater per well was 1:10, as described by Pinto et al.[22] Treatments were made simultaneously to the cyst immersion into the water, and the results were evaluated after 48 hours, corresponding to an average of the nauplii energetic autonomy time. From the results previously obtained on extract toxicity, extract 5 was chosen to be included in the seawater, 2.5 µg/L, the final STX concentration. Samples were analyzed in sextuplicate; the sum of cysts from each row of wells was considered the experimental unit. The total number of evaluated cysts was 6,594, averaging 5.1 per well. A descriptive semi-quantitative statistical analysis was used.

The results of this homeopathy screening test were evaluated using simple descriptive statistical analysis to select those potencies presenting promising protective-like effects based on the cyst hatching rate and living–dead nauplii/cysts ratio. In the second step, the leading tests were performed in three independent series, each in duplicate, to validate the selected treatments using a complete experimental set of statistical analyses. The experimental procedure was the same as described above, and the experimental unit was the sum of cysts from the same row of wells.

At this stage, the chosen treatments based on the preliminary screening were (see Results):

- *Plumbum metallicum* 6cH.
- *Nitric acidum* 6cH.
- Isotherapic (*R. raciborskii* extract) 200cH.

The three selected homeopathic preparations were compared to one another and the controls, according to cyst hatching and nauplii vitality (living nauplii/cysts ratio) rates after 48 hours. After observation, the content of microplates (water and biological sediment) was frozen at −20°C for additional gene expression and physicochemical analyses. At this step, the total number of evaluated cysts was 2,277, an average of 6.5 per well.

### *HSP* Gene Expression Involved in *Artemia franciscana* Bioresilience

From the results obtained in the screening experiments, the necessity to perform a deep study on the bioresilience process was identified. Thus, the gene expression of specific HSPs has been reported as a critical element in controlling cyst hatching and embryo development of *Artemia* spp.[11–15] It was also investigated here.

Gene expression tests were carried out on the stored frozen biological samples in the screening experiments. In the first step, total RNA extractions were done using the TRIzol/Chloroform/Isopropanol method.[45] For this, the *A. franciscana* structures (sediment composed of cysts and born nauplii) were thawed and pooled according to each treatment to warrant enough quantity of RNA to proceed with the assays. Each pool, containing the sediment from 12 wells, was homogenized in 0.5 mL of TRIzol (MERCK, Whitehouse Station, NJ, United States) and mixed with 0.2 mL of chloroform (MERCK, Whitehouse Station, NJ, United States). Then, it was centrifuged at 4°C, 14,000 rpm, for 15 minutes (Eppendorf 5804R centrifuge, Hamburg, Germany), the water fraction was removed, and the RNA fraction was precipitated by adding 0.5 mL of absolute cold isopropanol (MERCK, Whitehouse Station, NJ, United States) up to 10 mL, at room temperature. Next, the samples were centrifuged again; the RNA pellet was washed in 75% ethanol and suspended in



0.02 mL of diethyl pyrocarbonate aqueous solution. The quality and pureness of the extracted RNA were estimated by spectrophotometry (NanoDrop 2000, Thermo Scientific, United States). The optical density was proportional to the RNA content: that is, OD 260-280 = (≥1.8) and OD 230-260 = (≥1.0). Finally, the purified RNA samples were frozen at −80°C until the HSP expression assays began.

For gene expression analyses of specific mRNA (*Hsp26*, *p26, Hsp40*, *Hsp90*),[12–15] 2 µg of total RNA was used for cDNA synthesis with MMLV RNAse H minus first strand cDNA synthesis Kit (Nova Biotecnologia, Cotia, Brazil), according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was carried out in a total of 10 µL, containing SYBR green qPCR Master Mix 2x (5 µL) (Nova Biotecnologia, Cotia, Brazil), specific primers (5 µM), and 60 ng of cDNA nuclease-free $H_2O$ in a *QuantStudio 3* Real-Time PCR (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gene expression was expressed as compared to control cells by the $^{\Delta\Delta}$CT method, using *Ef1α*, *At* and *α-tubulin* represented on the plate as housekeeping controls. The primer sequences (EXXTEND, Paulinia, Brazil) and PCR conditions are shown in ►**Table 1**.

### *Raphidiopsis raciborskii* Growth Under Different Homeopathic Treatments

A new screening of treatments was performed on *R. raciborskii* T3 strain cultures to evaluate the colony growth rate trends. Then, those treatments presenting promising results were chosen for a complete statistical analysis and further experimental repetitions to confirm the results.

The cyanobacteria *R. raciborskii* T3 strain was donated to our laboratory by JSY (LCF-FURG, Brazil). Culture samples were sent to the UNIP Research Center, São Paulo, Brazil, in sealed sterile tubes at room temperature. The cultures were replicated in the standard ASM-1 medium[46] and kept in a separate room at constant temperature (25 ± 1°C) and light cycle (14 hours light – 10 hours dark), provided by a cold white fluorescent light fixed in the ceiling. Both environmental parameters were automatically controlled during the whole experimental period. Cultures were kept in 500 mL Erlenmeyer containers covered with a hydrophobic cotton/gauze cushion and put on a high but open shelf to avoid accidental shaking. The frame had lateral limits protected by wooden walls with enough space at the top for ventilation.

The cultures were replicated monthly for maintenance, and the experiments were performed after 15 days following a new replication. When the cultures reached the exponential growth phase, they showed a typical yellowish color (►**Supplementary file 3**, available in the online version) and filamentous microscopic structures.

A screening assay was done to select the best treatments, using a 15-day culture as a starting point. Aliquots of 3 mL containing $5 \times 10^7$ filaments/L were distributed in 69 culture tubes (10 mL, transparent glass) covered with hydrophobic cotton but kept with an air column to allow enough oxygenation (►**Supplementary file 3**, available in the online version). Each treatment was performed in triplicate.

This bacterial concentration ($5 \times 10^7$ filaments/L) corresponds to the amount needed to produce 3.6 µg/L of STX, according to the standards previously defined,[42] by using the formula:

$$y = 0.073x - 1.5065$$

where

$y$ = STX concentration (µg/L) and $x$ = number of filaments/mL.

This STX concentration is close to the maximum limit allowed in reservoirs by the Brazilian Health Ministry (*MS* 2914/2011), which is 3.0 µg equivalent STX/L.[47]

**Table 1** Available *primers* for identifying *Artemia franciscana* HSPs are considered bioresilience markers

| Gene | Primer | 5′–3′ Sequence | Reaction conditions |
|---|---|---|---|
| ART_*Hsp26* | Forward | CGG AGG ATT TGG TGG TAT GAC | 95°C, 15 s; 58°C, 30 s; 72°C, 30 s |
| | Reverse | CCT CAA GGA CCC AGG AGT AG | |
| ART_*Hsp40* | Forward | GTG CAT CAG TTG AGC GTC AC | 95°C, 15 s; 59°C, 30 s; 72°C, 30 s |
| | Reverse | TGCTGAACCATTCCAGGAGC | |
| ART_*Hsp90* | Forward | GGT GTG GGT TTC TAT TCT GC | 95°C, 15 s; 59°C, 30 s; 72°C, 30 s |
| | Reverse | GCA GCA GAT TCC CAC ACA | |
| ART_*p26* | Forward | GCG CGG ATC CAC CAT GGC ACT TAA CCC ATG | 95°C, 15 s; 57°C, 30 s; 72°C, 30 s |
| | Reverse | CGC GCC TCG AGT TAA GCT GCA CCT CCT GTC T | |
| ART_*At* | Forward | GCA GTG GTC TAC AAG GTT TC | 95°C, 15 s; 60°C, 30 s; 72°C, 30 s |
| | Reverse | ATC AAA ACG AAG GCT GGC GGT G | |
| ART_*Ef1α* | Forward | TCG ACA AGA GAA CCA TTG AAA A | 95°C, 15 s; 60°C, 30 s; 72°C, 30 s |
| | Reverse | ACG CTC AGC TTT AAG TTT GTC C | |
| ART_α-tubulin | Forward | CTG CAT GCT GTA CAG AGG AGA TGT | 95°C, 15 s; 60°C, 30 s; 72°C, 30 s |
| | Reverse | CTC CTT CAA GAG AGT CCA TGC CAA | |

Abbreviations: Hsp, heat shock protein; qPCR, quantitative polymerase chain reaction.
Note: Primers were used in the RNA expression assay (qPCR).

The counting of filaments was performed three times a week for 3 weeks to build a growing curve. Counting was made using a conventional Neubauer chamber, in which the number of filaments/mL was calculated using the following formula:

filaments/mL = 2500$y$

where

$y$ = sum of filaments counted in the four leukocyte quadrants (0.4 $mm^3$).

For the treatments, 0.3 mL (10% of the total) of each homeopathic preparation was poured into each tube once a week. The homeopathic preparations followed the same manipulation standards as described above (Toxicity of *R. raciborskii* Extract on *Artemia franciscana* Cyst Hatching). Baseline (no treated cyanobacteria), autoclaved pure water and autoclaved succussed pure water were used as controls, prepared as described above. All cyanobacteria manipulation was done in a laminar flow cabinet to prevent contamination.

In the first screening, the 22 homeopathic potencies were tested for hatching rate and nauplii vitality (ratio of number of live-born nauplii/cysts). A semi-quantitative evaluation of the whole set of parameters was done to select the treatments that presented the most convincing performance, considering the evidence of protection or "bioresilience". Then, the chosen treatments were tested again in the main experimental set.

In this screening, the treatments were randomly numbered by a person not involved in the experiment by changing labels by chance to those coded as letters (A, B, etc.). The original labels were fixed on a sheet of paper close to the respective code, which was saved in an envelope up to the end of the experimental procedures, when the codes were broken. All experiments were done blinded, and the codes were revealed after the tables and graphics were built. The treatments were:

- *Sulphur*—6cH, 30cH, 200cH.
- *Zincum metallicum*—6cH, 30cH, 200cH.
- *Nitric acidum*—6cH, 30cH, 200cH.
- *Plumbum metallicum*—6cH, 30cH, 200cH.
- *Mercurius solubilis*—6cH, 30cH, 200cH.
- *Phosphoric acidum*—6cH, 30cH, 200cH.
- Isotherapeutics prepared from *R. raciborskii* extract 5—6cH, 30cH, 200cH.

The controls were:

- Unchallenged, non-treated cyanobacteria (baseline);
- Hydrochloric acid 1cH ($5 \times 10^{-4}$ M).
- Succussed autoclaved pure water.

The protocols representing the most promising effect regarding cyanobacteria growth control were chosen to be used later, in the main experimental set.

They were:

- *Nitric acidum* 200cH.
- *Mercurius solubilis* 30cH.

The controls were:

- Succussed autoclaved pure water.
- Baseline (no treated cyanobacteria).

The main experimental set was carried out using the same protocol described for the screening phase, being $N = 9$: that is, nine tubes for each chosen treatment or control (total $N = 36$ tubes). In this case, the initial number of filaments was $20 \times 10^7$ per liter, corresponding to 14.6 µg/L of STX, to challenge the effectiveness of treatments in the worst conditions, with a significant population of *R. raciborskii.*

### Statistical Analysis

Statistical analysis and graphics were performed using GraphPad Prism version 9.5 for Windows. Normality was assessed by the Shapiro–Wilk test and by inspection of quartile-quartile (Q-Q) plots. One-way analysis of variance (ANOVA) evaluated normal variables for identifying statistical significance among treatments, and two-way ANOVA identified statistical significance among treatments as a function of time. Tukey's post-hoc test was used to compare one specified group to another. Following Tukey's rule, outliers were identified (using the Prism 9.5 tools) and removed if necessary. The significance level adopted was $\alpha = 0.05$.

## Results

### Toxicity of *Raphidiopsis raciborskii* Extract on *Artemia franciscana*

The hatching rate at 24, 48 and 72 hours after cysts' exposure to the extracts is shown in ►**Table 2**. Extract 5 caused moderate toxicity, not enough to kill all embryos but strong enough to disturb the cyst hatching rate: it was therefore the ideal condition with which to proceed with the following tests.

From this first result, the second assay was performed. Cyst hatching and nauplii death rates were calculated after 48 hours from different extract 5 concentrations in seawater, from 50 to 3.125%. Hydrochloric acid 0.05M and seawater were used as controls. These results are shown in ►**Table 3**.

The chosen working dilution of extract 5 was 12.5%, whose STX concentration is 2.5 µg/L. This concentration seemed quite strategic since the World Health Organization and the Brazilian Health Ministry (*MS* 2914/2011) adopted a 3.0 µg/L limit as the maximum acceptable level in public reservoirs.[47] Thus, this working concentration was considered an ideal experimental condition since it was strong enough to produce measurable changes in *A. franciscana* survival and, at the same time, it is related to real-world conditions. The environmental conditions registered in this step were temperature: 22.6 ± 1.94°C; humidity: 60.25 ± 5.06%; magnetic flux: 0.01 µT (non-variable).

#### Screening of Homeopathic Potencies

Reduction in hatching and increased vitality of nauplii indicate species-specific bioresilience attributes of *Artemia* spp. In this trial, the best performance was seen after the following treatments (in ascending order of their effectiveness):

**Table 2** Hatching rate of cysts after 24, 48 and 72 hours of incubation according to different *R. raciborskii* extract batches, whose concentrations were calculated according to the number of filaments produced by the cyanobacteria *in vitro*

| Challenge | Equivalent saxitoxin per well (µg/L) | 24 h hatching rate (%) | 48 h hatching rate (%) | 72 h hatching rate (%) |
|---|---|---|---|---|
| Baseline | N/A | 9.82 | 80.00 | 80.37 |
| Extract X | 69.42 | 9.40 | 85.00 | 85.23 |
| Extract Y | 42.46 | 0 | 0 | 0 |
| Extract Z | 21.25 | 0 | 0 | 0 |
| **Extract 5** | **20.86** | **5.71** | **39.00%** | **39.43** |
| Extract 6 | 16.08 | 0 | 1.00 | 0.85 |
| Extract 7 | 13.93 | 9.09 | 58.00 | 59.89 |
| Extract 8 | 05.69 | 8.60 | 73.00 | 78.49 |
| 1% hydrochloric acid 0.05M ($5 \times 10^{-4}$ M) | N/A | 3.95 | 54.00 | 64.47 |

Abbreviation: N/A, not applicable.
Bold font represents the chosen extract to be used in the subsequent experiments.

**Table 3** Cyst hatching and nauplii lethality rates (number of dead nauplii/total) after 48 hours of challenging the function of decrescent extract 5 concentrations in seawater

| Challenge | Saxitoxin concentration (µg/L) | Hatching rate (%) | Lethality rate (%) |
|---|---|---|---|
| 50% extract 5 | 10.0 (LC95) | 1.6 | 96.67 |
| 25% extract 5 | 5.0 (LC50) | 25.68 | 51.28 |
| **12.5% extract 5** | **2.5 (LC4)** | **70.59** | **4.00** |
| 6.25% extract 5 | 1.25 | 69.64 | 0 |
| 3.125% extract 5 | 0.6 | 72.41 | 0 |
| Hydrochloric acid (0.05 M) | N/A | 71.67 | 0 |
| Baseline 1 | N/A | 78.26 | 0 |
| Baseline 2 | N/A | 81.33 | 0 |

Note: The known saxitoxin concentration of the raw extract was 20 µg/L, according to the number of filaments produced by the cyanobacteria *in vitro*. N/A = not applicable. LC = lethal concentration able to kill 4, 50, or 95% of the nauplii.
Bold font represents the chosen concentration to be used in the subsequent experiments.

- *Nitric acidum* 6cH—low hatching rate and good viability, but low vitality.
- *Plumbum metallicum* 6cH—low hatching rate and best viability (0% death), but low vitality.
- Isotherapic 200cH—strong vitality and the best viability (0% death), but an unchallenged-like hatching rate. This was the best performance in the screening test.

Detailed results from the screening of potencies and the selection of those that showed optimal results on cyst hatching arrest and higher vitality of the born nauplii are described in ►**Supplementary file 4** (available in the online version).

Thus, the next step was a complete experiment, with statistical analysis, based on these three potencies, as described in Methods.

### Protection of *Artemia franciscana* from *Raphidiopsis raciborskii* Extract after Treatment with Homeopathic Potencies

A 15% lower rate of hatching of *A. franciscana* cysts was observed after treatment of challenged cysts with *Nitric acidum* 6cH in comparison with (unchallenged) baseline ($p = 0.03$) (►**Fig. 1**) but also reduced the live nauplii/cysts ratio (vitality) by about 16% ($p \leq 0.05$), showing worsening of the STX toxicity (►**Fig. 2**). However, Isotherapic (*R. raciborskii* extract) 200cH treatment again led to an unchallenged-like hatching rate (baseline), replicating the results of the screening phase. This latter effect is the most interesting since it preserves the natural behavior of nauplii during the exposure to cyanotoxins.

Succussed water and other treatments, including *Plumbum metallicum* 6cH, presented greater variance in baseline (coefficient of variation from 6 to 11%) than Isotherapic (*R. raciborskii* extract) 200cH (3.7% coefficient of variation) treatments. Also, Isotherapic 200cH presented no statistically significant differences in vitality in relation to baseline (►**Fig. 2**).

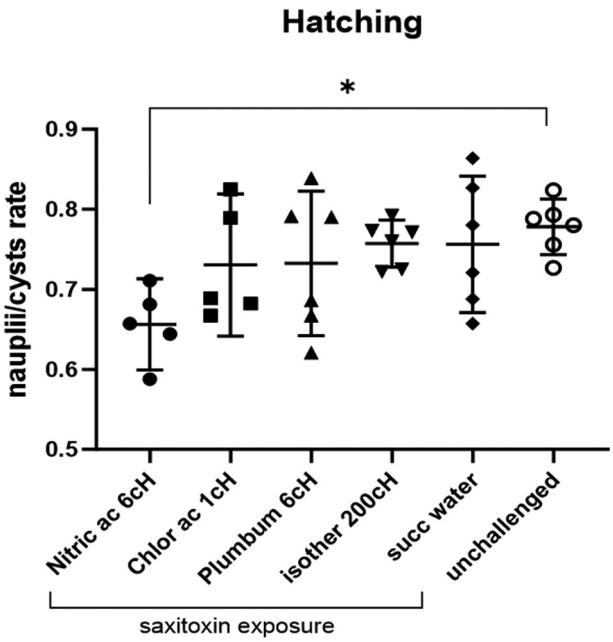
### HSP Gene Expression

The expression of the following genes was analyzed from the thawed biological material: *Hsp26*, *p26*, *Hsp40* and *Hsp90*. There was no statistically significant difference in *Hsp40* and *Hsp90* expression. However, compared with baseline, there was a 23-fold increase of *Hsp26* and a 24-fold increase of *p26* expression in the groups treated with Isotherapic (*R. raciborskii* extract) 200cH (►**Fig. 3**), indicating bioresilience improvement.

### *Raphidiopsis raciborskii* Growth after Treatment with Homeopathic Potencies

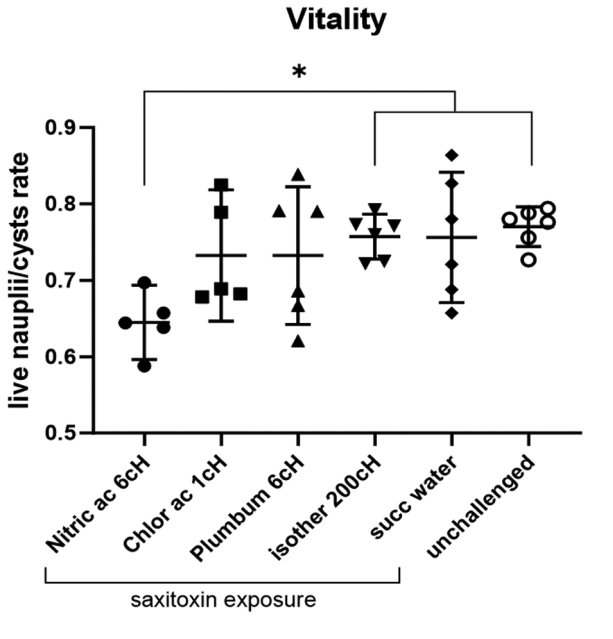
A screening test was performed to determine which homeopathic potencies were most promising in limiting the growth of cyanobacteria. *Nitric acidum* 200cH and *Mercurius solubilis* 30cH were chosen, showing a clear effect on cyanobacteria growth reduction (►**Fig. 4**). Detailed results from the




ANOVA, $F$ (5, 28) = 2.469, $p$ = 0.05, Tukey, * $p$ = 0.03

**Fig. 1** Cyst hatching rate after 48 hours of exposure to extract 5 (saxitoxin concentration = 2.5 µg/L) and treated with different homeopathic potencies. Unchallenged nauplii correspond to baseline. One-way ANOVA ($F_{(5, 28)} = 2.469$, $p = 0.05$; Tukey, $^{*}p = 0.03$). Values represent the mean and 95% confidence interval, and two outliers were identified. ANOVA, analysis of variance.




ANOVA, $F$ (2, 28) = 2.948, $p$ = 0.029, Tukey, * $p \leq 0.05$

**Fig. 2** Living nauplii/cysts rate (vitality) after 48 hours of exposure to extract 5 (saxitoxin concentration = 2.5 µg/L) and treated with different homeopathic potencies. Unchallenged nauplii correspond to baseline. One-way ANOVA ($F_{(2, 28)} = 2.948$, $p = 0.029$; Tukey $^{*}p \leq 0.05$). Values represent the mean and 95% confidence interval. Two outliers were identified. ANOVA, analysis of variance.

screening of potencies and the selection of those that showed optimal results on *R. raciborskii* growth control are described in ►**Supplementary file 4** (available in the online version).

In this screening test, cultures treated with *Nitric acidum* 200cH and *Mercurius solubilis* 30cH did not show growth peaks compared to baseline over two periods of exponential growth, with a growth limitation up to 95% and 85% respectively. Treatment with succussed water maintained a partially limited growth (about 50%) during the first peak of exponential growth, but this effect was not observed in the second peak. Such differences were statistically significant, presenting an interaction between time and treatment, $p < 0.0001$. This means that the treatment effect changed as a function of time, given the stationary and exponential phases observed during the experimental period (►**Fig. 4**).

The second test checked the previous results in more challenging conditions, with more repetitions. The results are shown in ►**Fig. 5**. In this second round, a late peak of exponential growth was seen only in the unchallenged culture (baseline), and the three treatments (*Nitric acidum* 200cH, *Mercurius solubilis* 30cH and *succussed water*) were equally capable of inhibiting the growth of *R. raciborskii*.

Comparing both graphs, the synchronism between the beginning of the treatment and the beginning of the exponential growth phase of *R. racibosrkii* seems to be another key factor in yielding clear evidence of growth inhibition.

## Discussion

Cyanobacteria blooms in aquatic environments are a real and worrying problem worldwide, affecting water quality and causing potential toxicity, with about 40 genera capable of generating cyanotoxins and producing accidental poisoning in animals and humans, chronically or acutely.[48,49] There is evidence that this is a growing problem, given the increasing incidence of water pollution in natural reservoirs.[49] This panorama inspires and justifies the present research.

Eutrophication, a phenomenon related to the enrichment of nutrients in water bodies by natural forces, refers to the increase in the amount of phosphorus, nitrogen and turbidity in water, and the pollution caused by organic waste. One of the consequences of eutrophication is the occurrence of exacerbated cyanobacterial blooms in various parts of the world.[50–53]

It is known that homeopathy produces regulatory effects on cellular functions in living systems, from microorganisms to humans.[22,26,27,29,54] Given this finding, the plausibility of using homeopathic potencies to facilitate the bioresilience of ecosystems and promote global health (within the "One Health" approach of the Food and Agriculture Organization of the United Nations [FAO]) is proposed. This approach does not address human health without addressing animal and environmental health simultaneously.[55] Given its current relevance, the "One Health" aim has been considered a priority by the FAO.[56] Consequently, studies on "homeopathy in environmental health" have been the focus of interest for our group in recent years, using brine shrimp as an experimental model, given its natural capacity for bioresilience.[22–24]

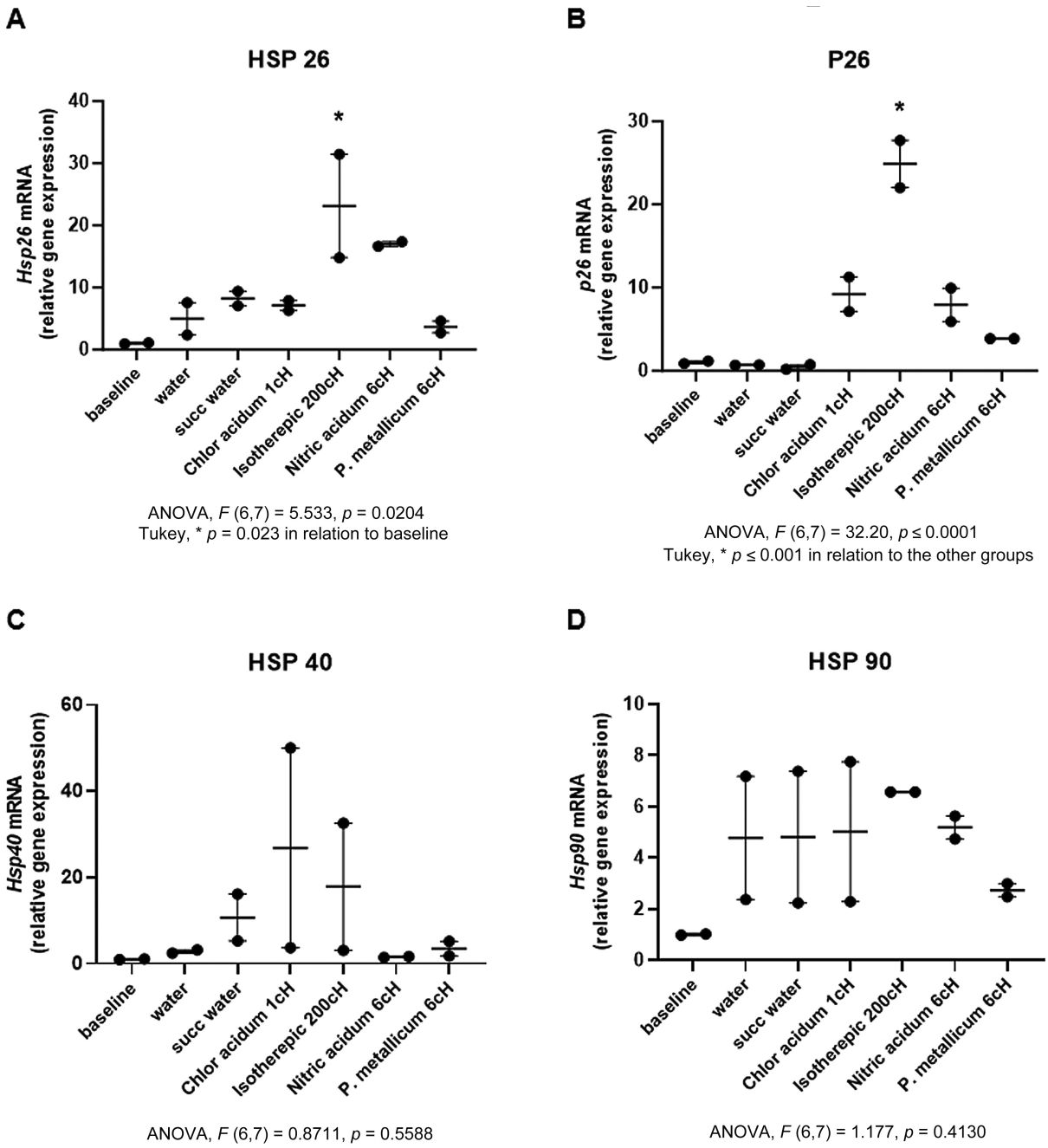



**Fig. 3** *Hsp26*, p26, *Hsp40* and *Hsp90* expression in embryo and nauplii exposed to extract 5 of *R raciborskii* (saxitoxin concentration = 2.5 µg/L) and treated with different homeopathic potencies. Unchallenged nauplii correspond to baseline. Statistical data are described at the bottom of each graphic. One-way ANOVA, followed by Tukey's post-test. Values represent the mean and standard error. No outliers were identified. ANOVA, analysis of variance.

In the present study, we used three inter-related experimental approaches to verify the plausibility of homeopathy to improve the condition of water-containing cyanobacterial colonies. The first was focused on the question: "Could homeopathic potencies improve the resilience of brine shrimp exposed to cyanotoxins?". The second aimed to answer the question: "Could homeopathic potencies regulate the growth of cyanobacteria even in a favorable environment for their growth?". The third question—the topic of our Part 2 paper[40]—was explicitly aimed at least partially to elucidate the involved mechanisms: "Is there a correspondence between biological effects and physicochemical changes in water after treating them with homeopathic potencies?".

To answer these questions, a well-known standard cyanobacteria strain (*R. raciborskii* T3 strain) was used as a model system, as it is well documented and described in terms of its growth in laboratory and natural conditions as well as the pharmacology of its neurotoxin.[1–6,18,20,57–59] Extract number 5 was previously obtained with a known STX concentration (20.86 µ/L STX equivalents). After a series of pilot tests with extract 5 dilutions and *A. franciscana*, the concentration of 2.5 µg/L STX equivalents (12.5% extract 5) was chosen to

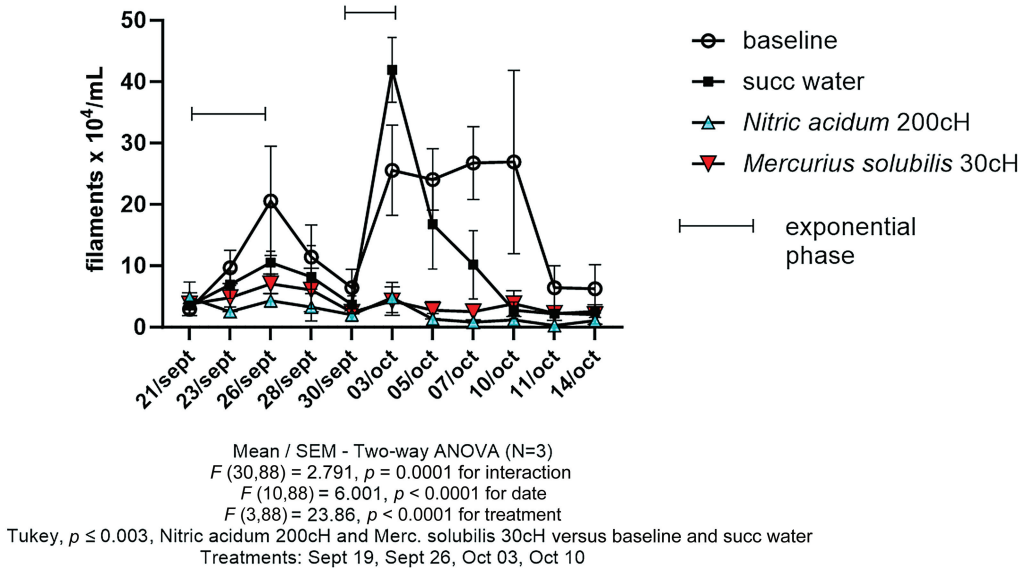



**Fig. 4** Growth of *R raciborskii* cultures (first round) in the function of the time comparing the chosen treatments (*Nitric acidum* 200cH and *Mercurius solubilis* 30cH) with succussed water and unchallenged/untreated cultures (baseline). Cultures started from a population of $5 \times 10^7$ filaments/L, able to produce 3.6 µg/mL of saxitoxin. Statistical data are described at the bottom of the graphic. Two-way ANOVA followed by Tukey's post-test. Values represent mean and standard error. Samples were done in triplicate, and no outliers were identified. ANOVA, analysis of variance.

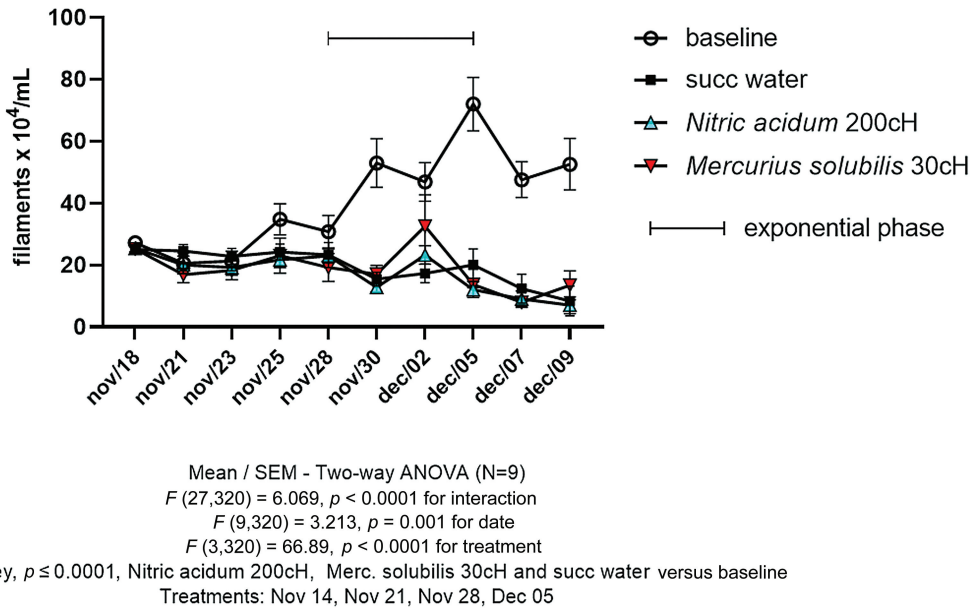



**Fig. 5** Growth of *R raciborskii* cultures (second round) as a function of time, comparing the chosen treatments (*Nitric acidum* 200cH and *Mercurius solubilis* 30cH) with succussed water and unchallenged/untreated cultures (baseline). Cultures started from a population of $20 \times 10^7$ filaments/L, able to produce 14.6 µg/mL of saxitoxin. Statistical data are described at the bottom of the graphic. Two-way ANOVA followed by Tukey's post-test. Values represent mean and standard error. $N = 9$ cultures per treatment. One outlier was identified on Nov/25. ANOVA, analysis of variance.

define a standard STX concentration close to the safety limits (3.0 µ/L) defined by the Brazilian Health Ministry,[47] to be used in the following stages of this study.

As for enhancing *A. franciscana* embryo bioresilience by homeopathic potencies, three samples showed interesting preliminary results in reducing cyst hatching rate. This is a protective response by the embryos by prolonging the diapause period.[11–13] However, the most promising result was observed after treating the cysts with Isotherapic (*R. raciborskii* extract) 200cH prepared from extract 5 of *R. raciborskii*, both in the screening phase and in the main experiment. In this case, the cysts hatched at the same rate as the non-exposed "baseline" group. This baseline group of cyanobacteria cultures represents the unique condition where no treatment or interventions were used, and bacteria have grown spontaneously over time. An apparent periodic curve is seen, which is expected for this species when cultivated in ASM-1 medium. Cyanobacteria grow in time-dependent serial cycles, faster or slower depending on the environmental conditions.[7]

Moreover, the vitality of the born nauplii was also comparable to "baseline". Vitality was defined as the number of live nauplii capable of swimming continuously in relation to the number of cysts. This result denotes an extraordinary level of bioresilience, in which the natural behavior of the nauplii was preserved even after exposure to cyanotoxins. Therefore, it was necessary to investigate the mechanisms involved through gene expression of HSPs, which actively participate in bioresilience processes in the genus *Artemia*.[11–15,60–63]

Compared to the other treatments, an increase in *Hsp26* and *p26* gene expression was seen in cysts and nauplii treated with Isotherapic (*R. raciborskii* extract) 200cH. The protein *p26* is a small HSP that is abundant in *A. franciscana* embryos during diapause. It is responsible for prolonging that quiescent stage, increasing the tolerance of embryos to stress.[60,61] Thus, a putative role of *p26* in selecting adapted embryos, and producing adapted nauplii to the presence of STX in water, was considered. The high level of *p26* expression suggests a narrowed role of *p26* in this selection after the treatment with Isotherapic 200cH. In this case, only the good responders were converted to healthy nauplii. A similar result was described previously.[31] Although the mechanisms involved are still unknown, the findings are consistent with the quantum electrodynamic hypothesis, as described by Madl & Renati, 2023.[30]

HSPs are present in all living beings and play a fundamental role in cell signaling and adaptation processes, especially as chaperones, whose function is to help proteins in the folding process: that is, in defining their three-dimensional tertiary structure, ensuring that they achieve the correct spatial design for their functions.[64,65] The protein *p26* belongs to a chaperone group that forms large oligomeric complexes, or *Hsp26*. Dissociation of the *Hsp26* complex is a prerequisite for the activity of these chaperones.[61,62] The viability and vitality of the born nauplii, therefore, could be an indirect consequence of the efficiency of these chaperones in the embryonic phase, generating larvae that are better able to survive despite the hostile environment.

Regarding the limitation of the exponential growth of *R. raciborskii* in ASM-1 medium, *Nitric acidum* 200cH and *Mercurius solubilis* 30cH showed a similar and lasting effect, regardless of the cyanobacterial population and the corresponding STX concentration. However, succussed water has a transitory impact due to the multiple growth cycles of cyanobacteria as a function of time. ASM-1 is ideal for facilitating its growth, containing various metals such as iron, copper, cobalt, molybdenum, sodium and potassium. The presence of these metals in water is a factor that favors cyanobacteria growth; for this reason, their presence and flowering are often an indicator of pollution.[48–53]

The quantitative evaluation of the growth rate was made from the filament count. Cyanobacterial filaments are like multicellular organisms, as nitrogen and carbon are exchanged among cells through the filament's septal junctions, ensuring their continuous growth. Filament counting is a commonly used technique to observe the growth rate of cyanobacteria.[46,47] In the exponential phase, for example, these microorganisms are at their maximum growth capacity, and the nutrient supplies of ASM-1 are more than sufficient for their needs.

It is understood that the efficiency of these homeopathic potencies under laboratory conditions suggests that they may also have potential in aiding the recovery of natural conditions, promoting the maintenance of water reservoirs in a sustainable way. Such limitation of cyanobacterial growth would not prevent their photosynthesis nor generate chemical residues in the water since the indicated potencies are prepared at concentrations beyond the Avogadro limit. The expected benefit would be to improve water quality for human and animal consumption, especially if associated with other sustainable water de-pollution methods. Therefore, the results obtained here inspire future studies in a field situation, together with contributing a new physico-chemical approach to understanding several biological features, as seen in the recent literature.[66–71]

In short, the results obtained in this study indicate the potential of homeopathic potencies to mitigate environmental problems related to microorganisms that impact ecosystems. This would contribute toward the FAO's recommendation for a "One Health" approach[55,56] and the sustainable development goals for the coming decades.[55] Physico-chemical experiments concerning the putative mechanisms involved in these reported results are presented in Part 2 of the study.[40]

## Conclusion

Isotherapic (*R. raciborskii* extract) 200cH proved to be the best option to improve the bioresilience of *A. franciscana* to STX, given its effects on cyst hatching, the vitality of born nauplii and *Hsp26*/*p26* expression. *Nitric acidum* 200cH and *Mercurius solubilis* 30cH were the optimal agents limiting the exponential growth of *R. raciborskii*. The results point to the potential of homeopathic potencies in mitigating environmental problems related to water quality.

### Highlights

- Cyanobacteria is a worldwide microorganism that threatens water quality.
- The putative mitigation of growth and toxicity of *R. raciborskii* with homeopathy was verified.
- The bioresilience of *A. franciscana* to STX was improved using Isotherapic 200cH.
- *Nitric acidum* 200cH and *Mercurius solubilis* 30cH reduced the *R. raciborskii* exponential growth.
- The helpfulness of homeopathy to mitigate some environmental problems was demonstrated.

## Supplementary Material

**Supplementary file 1.** *R. raciborskii* filaments quantification.
**Supplementary file 2.** The Faraday cage structure.
**Supplementary file 3.** *R. raciborskii* cultures.
**Supplementary file 4.** Screening of potencies.

Author Attributions

S.N.M. was the main researcher, PhD student, involved in all experimental procedures.
A.A.G.P. was responsible for experimental procedures—toxicity and solvatochromic dyes tests.
R.A.S. was responsible for experimental procedures—gene expression tests.
I.B.S. was responsible for experimental procedures—solvatochromic dyes tests.
A.T. was responsible for discussion of results on physicochemical parameters.
S.J.C. was responsible for experimental design with solvatochromic dyes and discussion of results.
J.S.Y. was co-adviser, providing cyanobacteria standards, and was responsible for the discussion of results.
L.V.B. was the main adviser and was responsible for the coordination of all steps of the study and discussion of results.

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Conflict of Interest

None declared.

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