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Environmental Homeopathy: Homeopathic Potencies Regulate the Growth and Toxicity of *Raphidiopsis raciborskii* (Cyanobacteria) and can be Tracked Physico-Chemically. Part 2: Physico-chemical Results

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Abstract

Introduction The control of cyanobacterial toxicity and growth by homeopathic potencies was described in Part 1 of this two-part report. Here, a parallel approach characterized the physico-chemical features of the potencies used and the liquid media treated with them, correlating these results with their respective biological effects.

Objectives Our objective was to establish if physico-chemical parameters can track homeopathic potencies in seawater or artificial seawater medium (ASM)-1 and to discover whether these parameters correlate with previously described biological effects.

Method *Artemia franciscana* (brine shrimp) cysts were cultivated in seawater challenged with *Raphidiopsis raciborskii* extract and treated with different homeopathic potencies chosen from a screening process. Cultures of *R. raciborskii* maintained in ASM-1 were also treated with previously screened homeopathic potencies, and their growth was monitored as a function of time. The physico-chemical properties of the treated media (seawater or ASM-1) were evaluated by their interaction with solvatochromic dyes and changes in pH, conductivity and temperature.

Results Coumarin 7 was found to be a marker for *Nitric acidum* 6cH and Isotherapic (*R. raciborskii* extract) 200cH in seawater (analysis of variance [ANOVA], $p = 0.0015$). Nile red was found to be a marker for *Nitric acidum* 200cH and *Mercurius solubilis* 30cH in ASM-1 (ANOVA, $p \leq 0.001$). An increase in pH of ASM-1 and endothermic effects were observed after these treatments (two-way ANOVA, $p = 0.0001$). Seawater and ASM-1 to which potencies had been added were also subjected to a constant unidirectional 2,400 Gauss static magnetic field and found to have enhanced effects on the solvatochromic dyes tested.

Keywords
- water
- solvatochromic dyes
- physico-chemical markers
- homeopathy

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Conclusion Homeopathic potencies were specifically traceable in aqueous media using solvatochromic dyes, especially when the samples were subjected to a magnetic field. Results from monitoring other physical parameters, such as pH and temperature, were less specific in relation to potency tracking. However, potency-induced endothermic effects might provide valuable thermodynamic data relating to the nature of potencies.

Introduction

In a previous study, reported in this issue,[1] Isotherapeutic (*Raphidiopsis raciborskii* extract) 200cH was the best option to improve the bio-resilience of *Artemia franciscana* (brine shrimp) to saxitoxin, as shown by its effects on cyst hatching, nauplii vitality and *hsp26*/*p26* gene expression. The *hsp 26* and *p26* genes code for heat shock proteins, which can be considered bio-resilience markers for *Artemia sp*.[2–10] Additionally, *Nitric acidum* 200cH and *Mercurius solubilis* 30cH were the best agents limiting the exponential growth of *R. raciborskii* in artificial seawater medium (ASM)-1,[1] a rich medium considered ideal for cyanobacteria growth in laboratory conditions.[11]

These results, taken together, highlight the potential of homeopathy in mitigating environmental problems related to water quality. Previous studies have shown potency-mediated bio-resilience of *Artemia salina* to mercuric chloride[12] and other toxic agents in seawater,[13,14] corroborating this perspective.

Given that such homeopathic dilutions often exceed Avogadro's limit, it is necessary to explore possible 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.[15–17] Furthermore, such biological effects have shown close correspondence with physico-chemical changes in previous microcrustacean studies.[12] These physico-chemical changes can be monitored through interactions with solvatochromic dyes.[18–25] A recent systematic experimental study has also observed correspondence between biological effects and physico-chemical changes.[26]

Solvatochromic dyes have been found to be probes able to track homeopathic potencies both in the laboratory[18–21,23–26] and in field studies in the natural environment.[22] The nature of the interaction between solvatochromic dyes and homeopathic potencies is yet to be elucidated, but the evidence so far indicates that the unique electronic structure and behavior of these dyes is crucial to the interaction and that the interaction involves an increase in their polarity, which can be measured spectroscopically in the UV-visible region of the electromagnetic spectrum.[24] Increased polarity associated with high dilution activity has been reported since the 1980s,[27–31] which leads to the following hypothesis:

Since homeopathic remedies are made in dipole bases (water, alcohol, lactose, saccharose) and solvatochromic dyes are dipoles too, changes in the dipole moment of the solvent used to prepare the dyes can be observed after the addition of specific homeopathic potencies, in such a way that solvatochromic dyes can act as probes to detect those changes by modifying their color intensity. Indeed, this effect can be reflected in the absorbance measurement using a spectrophotometer, as seen previously.[18–26] In addition, a static magnetic field, being strong enough, can *a priori* provide a more stable magnetic interaction between dye and solvent molecules[24] through the alignment of the dipoles so that sharper differences between homeopathic potencies and controls may be identified. This fact would imply an increase in the method's sensitivity. Therefore, other parameters related to dipole variations can also be applied as tracking systems: that is, pH, conductivity and temperature.

Based on the above, the present study sought to test the hypothesis that homeopathic preparations can be tracked in solution by using physico-chemical measurements such as pH, conductivity, temperature and interaction with solvatochromic dyes, in conjunction with their use in controlling the growth and toxicity of cyanobacteria. Solvatochromic dyes are sensitive to a number of environmental stimuli and solution conditions, including solvent polarity and levels of hydrogen bonding, as well as changes in ambient electrical field strengths.[20–25] Different solvatochromic dyes respond to homeopathic potencies according to their structure, so selective detection and identification of various homeopathic potencies may be possible.[18,19,26] The present study aims, among others, to see if this possibility can be realized.

The present study (the second of a two-part series[1]) is driven by the need to look for cheap and effective technologies that could track the presence of homeopathic potencies in water under natural, real-life conditions. An initial study showed relevant results in this field of investigation,[22] and this new study aims to extend those results in laboratory conditions.

Materials and Methods

Sample Analysis by the Solvatochromic Dyes Method

Samples of medicines selected in previous experiments,[1] samples of water from *Artemia franciscana* cultures and samples of *R. raciborskii* culture were used.

Sample Preparation

All samples followed a previously standardized preparation protocol.[26] In laminar flow, 10 mL of 30% ethanol diluted in

purified autoclaved water (SmartPak Direct Q3 with Biopak filters—Merck–Millipore, Darmstadt, Germany) was filtered through a 0.22-micrometer mesh filter (Merck–Millipore, Darmstadt, Germany) into a conventional type 2 amber glass flask. Then, 100 µL of previously succussed and filtered sample (Merck–Millipore, Darmstadt, Germany) was added to each flask to give a hundred-fold dilution. Flasks were closed and submitted to 100 succussions in an automatic robotic arm (Denise-Autic, São Paulo, Brazil).

Each medicine sample was prepared from the respective stock potency. Each water sample (ASM-1 or seawater) was prepared from the liquid content of a pool of wells submitted to the treatment described above and randomly analyzed in quadruplicate after thawing. Two kinds of pools were prepared for each treatment, each corresponding to a test step (screening or repetition).

The selected homeopathic potencies and controls followed the same screening procedure described in the connected Part 1 paper.[1] They were as follows:

- Water (baseline);
- Succussed autoclaved pure water (treatment control);
- Hydrochloric acid 1cH (5×10^{-4} M) (medium control);
- 30% ethanol (vehicle control);
- Isotherapic (*R. raciborskii* extract) 200cH;
- *Plumbum metallicum* 6cH;
- *Nitric acidum* 6cH;
- *Nitric acidum* 200cH;
- *Mercurius solubilis* 30cH.

The seawater samples obtained from *Artemia franciscana* experiments to proceed with the analysis using solvatochromic dyes followed the same screening procedure described in the connected research.[1] They were treated with:

- *Plumbum metallicum* 6cH;
- *Nitric acidum* 6cH;
- Hydrochloric acid 1cH;
- Isotherapic (*R. raciborskii* extract) 200cH;
- Succussed water;
- Water.

An additional control group, "Baseline" (from cultures neither challenged nor treated), was also tested.

The ASM-1 samples selected from cultures of *R. raciborskii* were those treated with the following:

- *Nitric acidum* 200cH;
- *Mercurius solubilis* 30cH;
- Baseline (from cultures neither challenged nor treated);
- Succussed water.

Sample Analyses

The dyes used were Coumarin 7, Nile red, N,N-dimethylindoaniline (NN-DMIA), 4-(bis-[4-[dimethylamine] phenyl methylene]-1(4H)-naphthalenone, dimethylamino-benzylidene-rhodanine and methylene violet (►**Supplementary file 1**, available in the online version), all diluted in absolute ethanol (Synth, Diadema, Brazil) according to the previously standardized ideal concentration,[12,20,21] 24 hours before carrying out the tests for stabilization. Before use, all dyes were filtered through a 0.22-micrometer mesh filter (Merck–Millipore, Darmstadt, Germany).

A preliminary test was conducted to screen those dyes capable of interacting with each homeopathic potency. The dyes that presented the most evident increase or decrease in absorbance were selected to analyze the *Artemia franciscana* or cyanobacteria culture samples.

Before adding to the dyes, potencies were again manually succussed using 40 vertical movements, mimicking the mechanical arm movements of the *Denise* device (AUTIC, São Paulo, Brazil), as used in the laboratory routine (semicircular movement, with a 55 degrees angle, 300 mm radius, with operational cycle equivalent to 100 pulses in 33 seconds, 1,620 rpm/880g, according to manufacturer information) and generating a calculated force of approximately 2,420 N. After this, they were filtered through a 0.22-micrometer mesh filter (Merck–Millipore, Darmstadt, Germany). All procedures were carried out in a laminar flow cabinet, whose environmental conditions were registered daily (temperature: 25.8–25.9°C; humidity: 39–43%; magnetic flow: 0.03–0.07 µT). Measurements were made with an thermo-hygrometer (Tomate PD-003, São Paulo, Brazil) and an electromagnetic field meter (frequency range: 30 to 300 Hz, resolution 0.01 to 0.1 µT, 3% precision at 50–60 Hz—Instrutherm DRE 050, São Paulo, Brazil).

The spectrophotometer used in the experiment (FEMTO 800 XI, São Paulo, Brazil), with a sensitivity of 1 nm, was calibrated with pure ethanol (Synth, Diadema, Brazil) to determine the baseline and used to scan the entire visible spectrum (350 to 800 nm) of each testing dye to identify the specific wavelength related to the absorbance peak. Thus, the sample analyses were performed in a microplate spectrophotometer (EPOCH – Agilent BioTek, Santa Clara, CA, United States), whose wavelengths were selected accordingly. This procedure was done in each experimental series. Data were automatically generated in an Excel electronic spreadsheet.

The homeopathic potency samples were distributed in conventional flat-bottomed 96-well microplates for enzyme-linked immunosorbent assay tests to screen responsive dyes in the first round. Each sample was distributed in four to eight wells per plate in two experimental series. In each plate, a row of eight wells containing only absolute ethanol and another containing only pure dye (diluted in alcohol) was prepared. In the following rows, 4 µL of the experimental and control samples were inserted into each well, containing 236 µL of dye, in a 1:60 ratio.[12,20,21] An example of a microplate plan and the sample absorbance calculation is illustrated in ►**Diagram 1**. The natural absorbance of absolute ethanol was discounted for calibration.

Tests were carried out in two stages, the first being as described above and the second carried out after submitting samples to a static magnetic field for 15 minutes, using a neodymium magnet model N42—NdFeB (Magnetum Produtos Magnéticos, São Paulo, Brazil), measuring $5.08 \times 5.08 \times 1.27$ cm, in a 270 g block format, coated with nickel, whose capacity is 2,400 Gauss, according to the manufacturer's information. The exposure time was chosen based on a

			Control samples (4 µl)			Verum samples (4 µl)								
	Ethanol PA	Pure dye	Water	Succ water	Chlor ac 1cH	Isother 200cH	Plumbum met 6cH	Nitric acidum 6cH	Nitric acidum 200cH	Merc solubilis 30cH	Void	Void	Void	Void	Weivelength
	1	2	3	4	3	4	5	6	7	8	9	10	11	12	
A	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528
B	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528
C	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528
D	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528
E	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528
F	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528
G	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528
H	a	D	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample	d + sample					528

a = pure alcohol for calibration (240 µl) D = pure dye in ethanol PA (240 µl) d = pure dye in ethanol PA (236 µl)

Pure dye absorbance (PDA) = D average - **a** average

Sample absorbance = d + sample absorbance - **PDA**

Diagram 1 Example of mapping sample distribution in an ELISA microplate and sample absorbance calculation. ELISA, enzyme-linked immunosorbent assay.

previous study[32] that demonstrated interference with homeopathic high-dilution biological effects (see Discussion for details).

In the magnetic treatment experiments, all samples of the same experiment were simultaneously subjected to the magnetic field. The microtubes were placed at the bottom of a beaker and positioned in the same direction of the magnetic flow, with the beaker bottom placed on the positive surface of the magnet, as shown in ►**Supplementary file 2** (available in the online version). To avoid interference, the magnet was set on a bench free of any equipment plugged into the electric current, and cell phones were kept at least 2 m away.

After analyzing the interaction between potencies and dyes, the most striking results were obtained following the exposure of samples to a static magnetic field. A more significant difference between controls and samples was seen, with more minor standard deviations. Exposure of samples to a static magnetic field was therefore chosen as the standard method for all following experiments involving the analysis of culture medium samples. The dyes selected for these analyses were: (1) Coumarin 7, responsive to the Isotherapic (*R. raciborskii* extract) 200cH, *Plumbum metallicum* 6cH and *Nitric acidum* 6cH; and (2) Nile red, responsive to *Nitric acidum* 200cH and *Mercurius solubilis* 30cH.

An illustrative chart of the experimental design is shown in ►**Fig. 1**.

Analyses of Physico-Chemical Parameters of *Raphidiopsis raciborskii* Cultures

Cultures of *R. raciborskii* in ASM-1 were used in this assay, as described.[1] Based on the results from that study in which *Nitric acidum 200cH* and *Mercurius solubilis 30cH* were found to inhibit *R. raciborskii* effectively, these potencies were taken and assessed physico-chemically. Succussed water was used as vehicle control, and data obtained from untreated cultures were considered baseline. The three preparations were blinded before the start of the experiment, and the codes were opened only after statistical analysis.

Samples of 100 mL of *R. raciborskii* culture in ASM-1 containing 3×10^7 filaments per liter were inserted into 250 mL beakers ($N = 4$ per treatment) and kept in cabinets with stainless steel walls and a glass side lid to allow light to pass through. Using those cabinets permitted the maintenance of a protected environment during the experiments. Cold white fluorescent light fixed in the roof provided the light cycle (12 hours light – 12 hours dark). Temperature (25.7 ± 0.74°C), humidity (65.8 ± 3.42%) and constant magnetic field (0.01 µT) inside the cabinets were recorded daily throughout the experiment.

Two cabinets were used simultaneously: one exclusively with cultures treated with potencies not subjected to the magnetic field and the other exclusively with cultures treated with potencies subjected to a magnetic field. The tests were carried out over 5 days. The physico-chemical parameters of the medium (temperature, pH and conductivity) were recorded daily using multi-parameter sensors (Waterproof pH/EC/temperature meter—JuanJuan Electronic Technology, Guangdong, China). Those sensors were calibrated simultaneously before the beginning of the test, using the same standard solutions, and made by two persons in a double-check system.

The experimental design was defined as follows: *Day 1*—The cultures were divided into 100 mL aliquots and distributed in 250 mL beakers, four aliquots per treatment. This way, 16 beakers were placed in each cabinet equally apart and covered with a filter paper lid to avoid the accidental deposition of particles suspended in the air (►**Supplementary file 3**, available in the online version). The first measurement of physico-chemical parameters was performed on this day. *Day 2*—The physico-chemical parameter measurements were performed twice on this day, the first time immediately before the potencies (or controls) were inserted into the medium and the second time immediately afterward. The treatments were performed by inserting 100 µL of each potency in the respective beaker. Only measurements of physico-chemical parameters were performed on *day 3*, *day 4* and *day 5*. Four meters

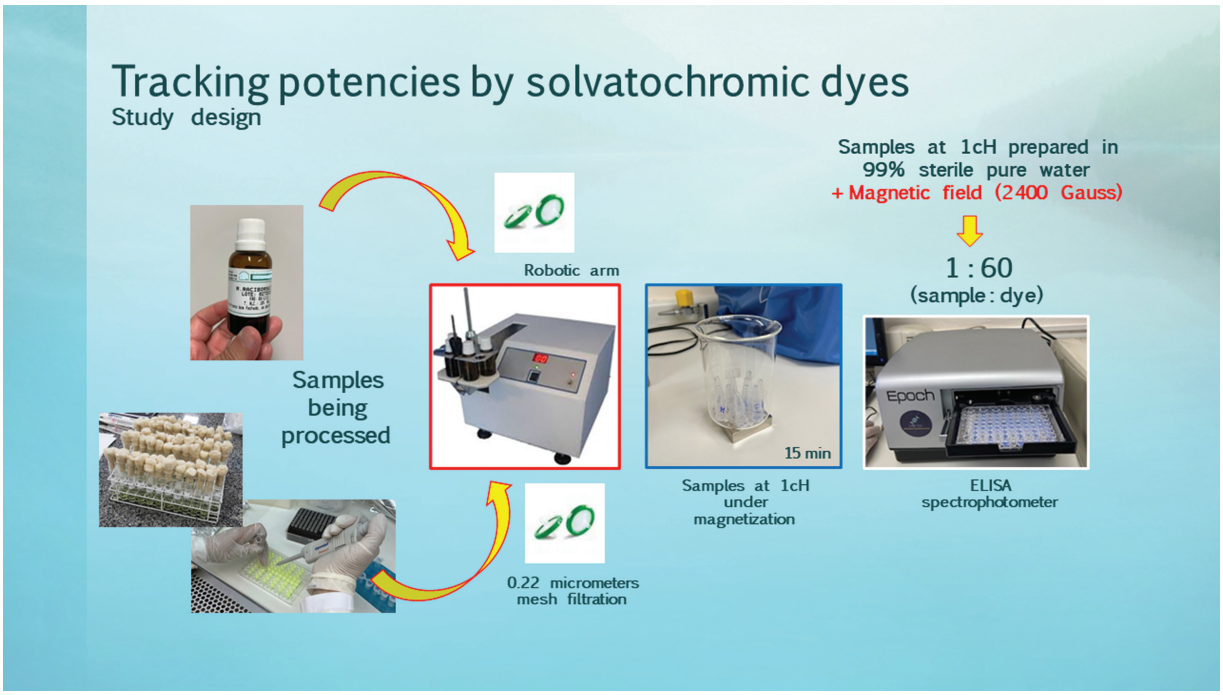

Fig. 1 Illustrative chart of the experimental design involving solvatochromic dyes interaction with homeopathic potencies and treated water samples.

were used, one for each treatment group, avoiding contamination between samples. Between one measurement and the other, the sensors were washed with purified autoclaved water (SmartPak Direct Q3 with Biopak filters—Merck–Millipore, Darmstadt, Germany) in a plastic cup and dried with soft tissue paper. Every day, before starting the experiments, the sensors were sterilized under an ultraviolet light inside a plastic envelope for 15 minutes. All measurements were made in the morning.

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 plots (Q–Q plots). One-way analysis of variance (ANOVA) evaluated Normal variables for identifying statistical significance among treatments and two-way ANOVA for identifying statistical significance among treatments as a function of time. Tukey's post-test was used to compare one group to another. Variables that did not fit the Normality test were evaluated by Kruskal–Wallis analysis, followed by Dunn's post-test. Following Tukey's rule, outliers were identified using the automatic Prism 9.5 tool for outlier identification and removed from the analysis if necessary. Outliers are represented as isolated dots in the graphics. The significance level adopted was $\alpha = 0.05$.

Results

Analysis of Homeopathic Potencies Using Solvatochromic Dyes

Following screening (►**Supplementary file 4**, available in the online version), *Plumbum metallicum* 6cH, *Nitric acidum* 6cH and Isotherapic (*R. raciborskii* extract) 200cH, used in treating *Artemia franciscana*[1] (in this issue), showed better interaction with Coumarin 7 and methylene violet in relation to the other dyes. This effect was sharper and more specific after submitting the samples to the magnetic field (►**Fig. 2**). Considering the whole data set, the method that best identified the homeopathic potencies compared to controls was the interaction of homeopathic potencies with Coumarin 7 when previously subjected to the magnetic field. Thus, Coumarin 7 was chosen for the evaluation of seawater samples.

Nitric acidum 200cH and *Mercurius solubilis* 30cH (►**Supplementary file 4**, available in the online version), used in treating *R. raciborskii* cultures,[1] showed a better interaction with Nile red than other dyes, and results were more precise and more specific after submitting the potencies to a magnetic field (►**Fig. 3**). In the whole data set, Nile red showed less variance and more striking results than NN-DMIA. Therefore, Nile red can be considered a suitable probe for *Nitric acidum* 200cH and *Mercurius solubilis* 30cH when previously submitted to a magnetic field.

Additionally, there was a clear correlation between the interaction of *Nitric acidum* 200cH and *Mercurius solubilis* 30cH with Nile red and the effects of these potencies on *R. raciborskii* growth, as shown previously.[1]

Analysis of Seawater and Artificial Seawater Medium Samples with Solvatochromic Dyes

After testing dyes with different potencies, Coumarin 7 was considered the ideal dye for analyzing treated seawater sample pools. Each pool corresponded to a row of wells. Two pools of samples were analyzed in quadruplicate and subjected to a constant magnetic field immediately before

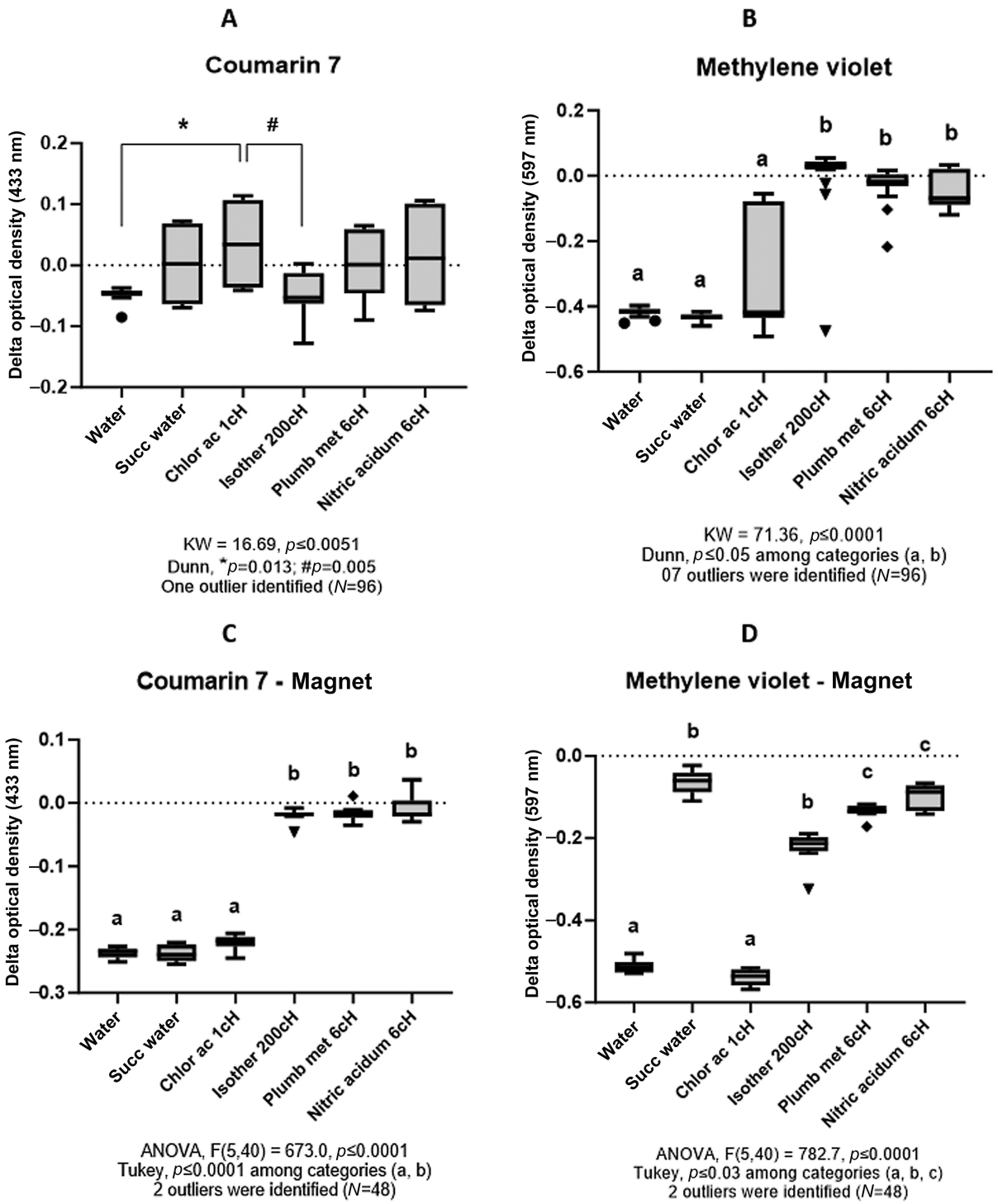

Fig. 2 Boxplot of coumarin 7 and methylene violet absorbance after interacting with Isotherapic (*R. raciborskii* extract) 200cH, *Plumbum metallicum* 6cH, *Nitric acidum* 6cH and controls. Kruskal–Wallis/Dunn was used in A and B since the variables presented non-Normality in the Shapiro–Wilk test. One-way ANOVA/Tukey was used in C and D since the variables presented Normality in the Shapiro–Wilk test. Statistical data are described at the bottom of the graphic, including the total N used in each analysis. The outlier ratio varied from 1 to 7%. Outliers are indicated as dots with different shapes. ANOVA, analysis of variance.

interacting with the dye. All homeopathic potencies were responsive to Coumarin 7 (ANOVA, $p = 0.0015$), including hydrochloric acid 1cH, as shown in ►**Fig. 4**.

Likewise, Nile red was the ideal dye for analyzing treated cyanobacterial culture medium (ANOVA, $p \leq 0.001$). The samples were analyzed in experimental quadruplicates for each study stage (screening and main experiment), as described.[1] Additional triplicates or quadruplicates were done for the spectrophotometric reading, resulting in $N = 12$ for the screening samples (series 1) and $N = 16$ for the main

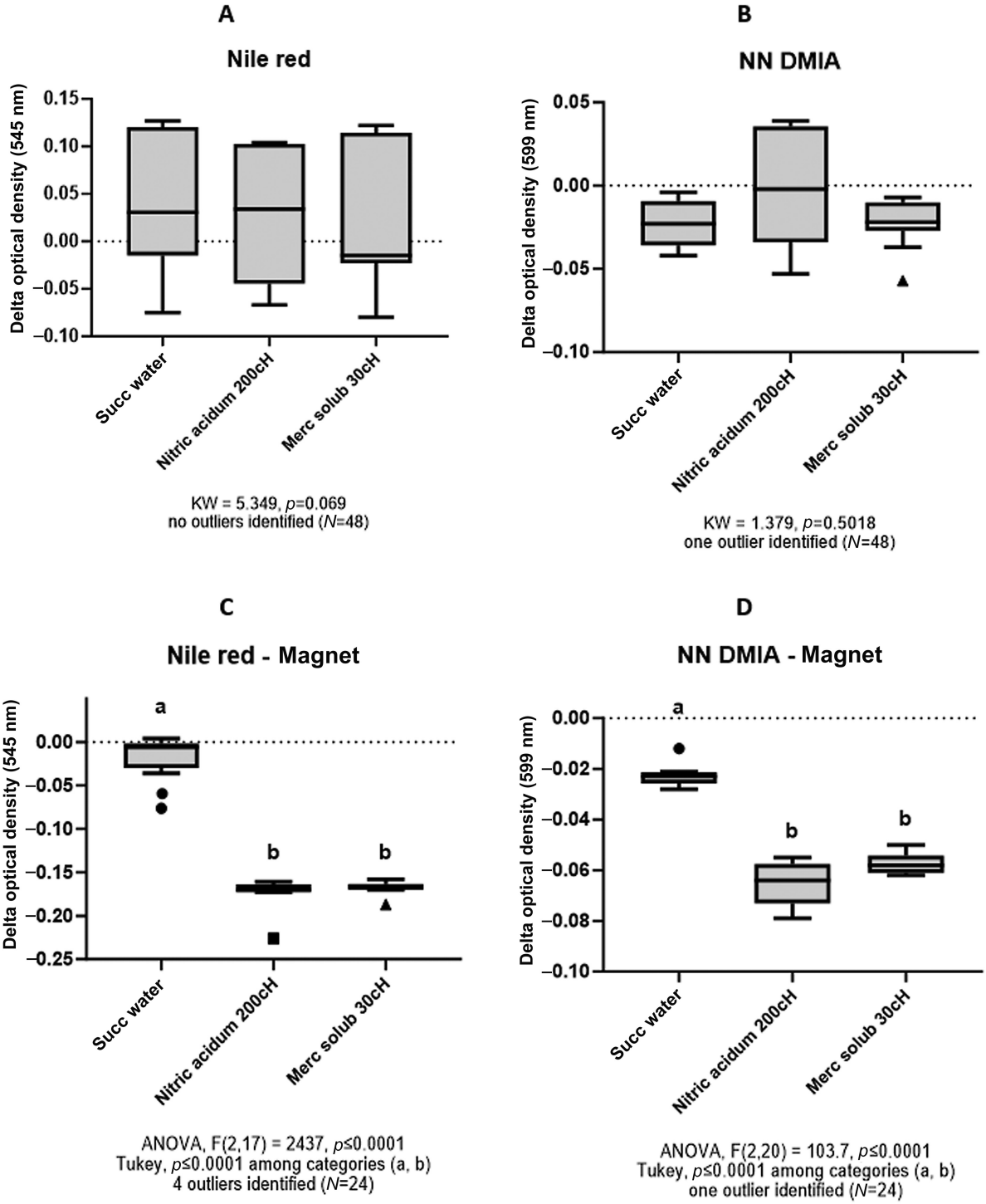

Fig. 3 Boxplot of Nile red and NN-DMIA absorbance after interacting with *Nitric acidum* 200cH, *Mercurius solubilis* 30cH and controls. Kruskal–Wallis/Dunn was used in A and B since the variables presented non-Normality in the Shapiro–Wilk test. One-way ANOVA/Tukey was used in C and D since the variables presented Normality in the Shapiro–Wilk test. Statistical data are described at the bottom of the graphic, including the total N used in each analysis. The outlier ratio varied from 1 to 16%. Outliers are indicated as dots with different shapes. ANOVA, analysis of variance; NN-DMIA, N,N-dimethylindoaniline.

experiment samples (series 2). The results can be seen in ►**Fig. 5A, 5B**.

Solvatochromic dyes respond to homeopathic potencies through an increase in their electronic polarization. Changes in their spectra reflect this increase in polarization, but absorbances can increase or decrease according to a dye's particular electronic structure and aggregation characteristics in solution.

In both experimental situations described in ►**Figs. 3** and **4**, the sample's response to the elected solvatochromic dye mirrored the biological effects on *Artemia franciscana* vitality or cyanobacteria growth, as shown earlier.[1]

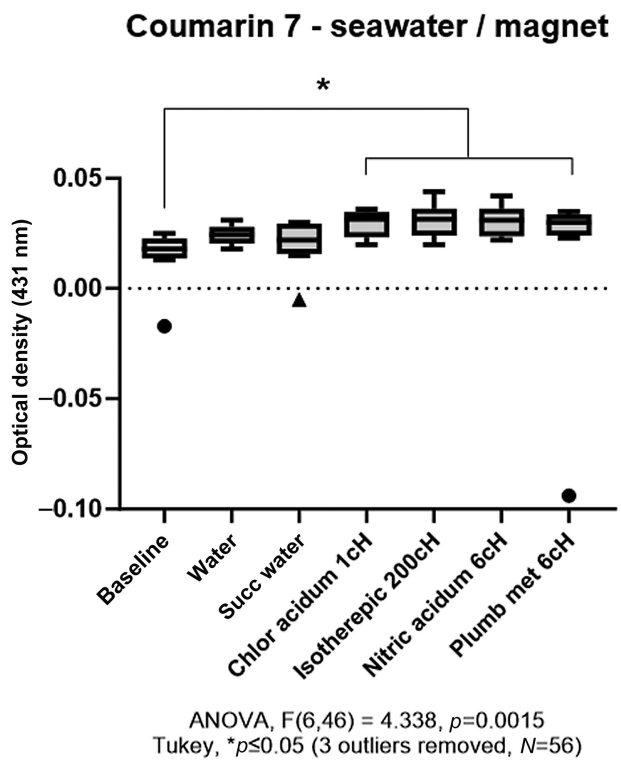

Fig. 4 Boxplot of Coumarin 7 absorbance after interacting with Isotherapic (*R. raciborskii* extract) 200cH, *Plumbum metallicum* 6cH, *Nitric acidum* 6cH, hydrochloric acid 1cH and controls. One-way ANOVA/Tukey was used since the variables presented Normality in the Shapiro–Wilk test. $F_{(6,46)} = 4.338$, $p = 0.0015$, Tukey $^{*}p \leq 0.05$. Data show the combination of two pools of water, analyzed in quadruplicate. The outlier ratio was 5%. Outliers are indicated as dots with different shapes. ANOVA, analysis of variance.

Analysis of Physico-Chemical Parameters of Artificial Seawater Medium before and after the Treatment of *Raphidiopsis raciborskii* with Homeopathic Potencies

No changes in conductivity were observed as a function of treatments and time (there was no statistical interaction between them), regardless of whether the homeopathic potencies were subjected to a magnetic field before being added to the cyanobacteria cultures, as shown in ►**Fig. 6**.

On the other hand, pH variations showed the need to stabilize cultures in the first 24 hours after distribution in the beakers to reach the expected pH range for ASM-1 (pH = 7.5–8.0). This was observed in both batches, batch 1 (used later for treatment with potencies *not* subjected to a magnetic field) and batch 2 (used later for treatment with potencies subjected to a magnetic field; ►**Fig. 7**). Then, it was possible to perform treatments and sequential observations as a function of time.

The pH curve in the different experimental conditions and treatments between days 2 and 5 showed a slight reduction in pH over the days, ranging from 7.9 to 7.7 in the "baseline" group of both batches, possibly associated with continuous bacteria growth (see Discussion section below). There was a sustained higher pH after the insertion of *Nitric acidum* 200cH and, to a lesser extent, *Mercurius solubilis* 30cH, in the culture medium relative to controls, with no statistical interaction between time and treatment (►**Fig. 8A**).

Submitting the homeopathic potencies to the magnetic field, in turn, resulted in a more precise and lasting difference between treatments and controls (two-way ANOVA, $p = 0.0001$). In this case, both *Nitric acidum* 200cH and *Mercurius solubilis* 30cH showed the same behavior, with greater statistical significance for *Nitric acidum* 200cH

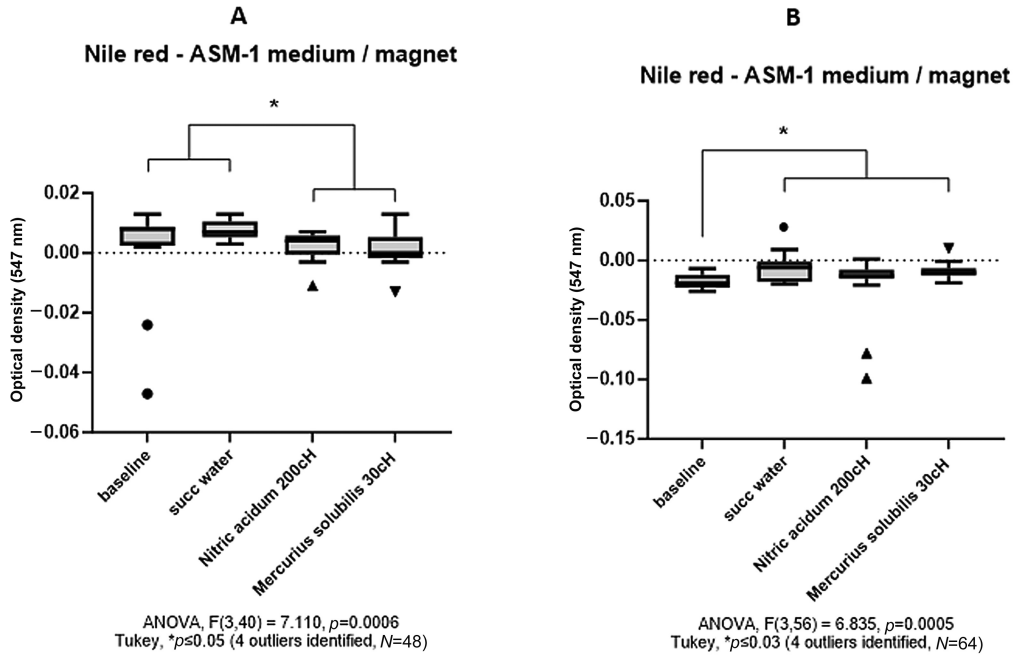

Fig. 5 Boxplot of Nile red absorbance after interacting with *Nitric acidum* 200cH, *Mercurius solubilis* 30cH and controls. One-way ANOVA/Tukey was used since the variables presented Normality in the Shapiro–Wilk test. **(A)** Samples obtained from the screening test (series 1), $F_{(3,40)} = 7.110$, $p = 0.0006$, Tukey $^{*}p \leq 0.05$, $N = 48$. **(B)** Samples obtained from the repetition test (series 2), $F_{(3,56)} = 6.835$, $p = 0.0005$, Tukey $^{*}p \leq 0.03$, $N = 64$. The outlier ratio was 6 to 8%. Outliers are indicated as dots with different shapes. ANOVA, analysis of variance.

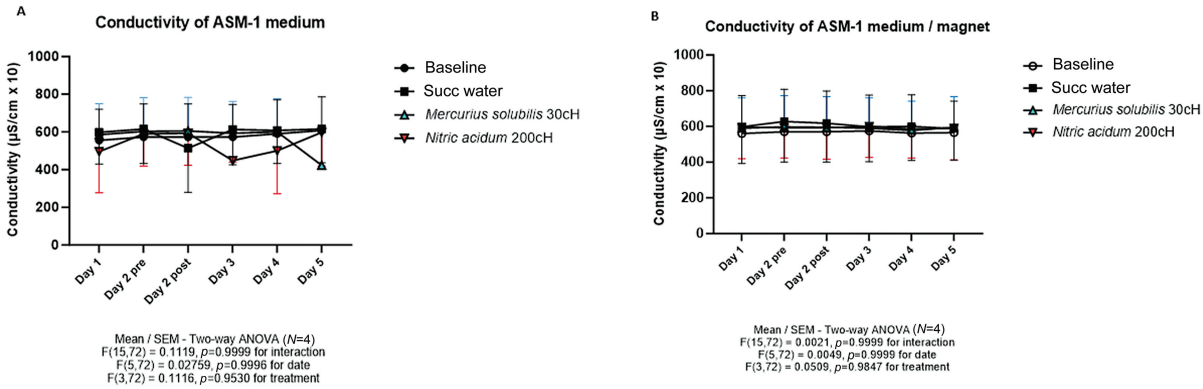

Fig. 6 Conductivity variation of *R. raciborskii* culture medium submitted to different treatments (*Nitric acidum* 200cH and *Mercurius solubilis* 30cH) and controls as a function of time. (**A**) The time-dependent curve of cultures treated with homeopathic potencies *not* subjected to the magnetic field. (**B**) The time-dependent curve of cultures treated with potencies subjected to a magnetic field immediately before immersion into the medium. Two-way ANOVA/Tukey was used since the variables presented Normality in the Shapiro–Wilk test. Statistical data are described at the bottom of the graphic. The mean and standard error represent the data generated in quadruplicates. ANOVA, analysis of variance.

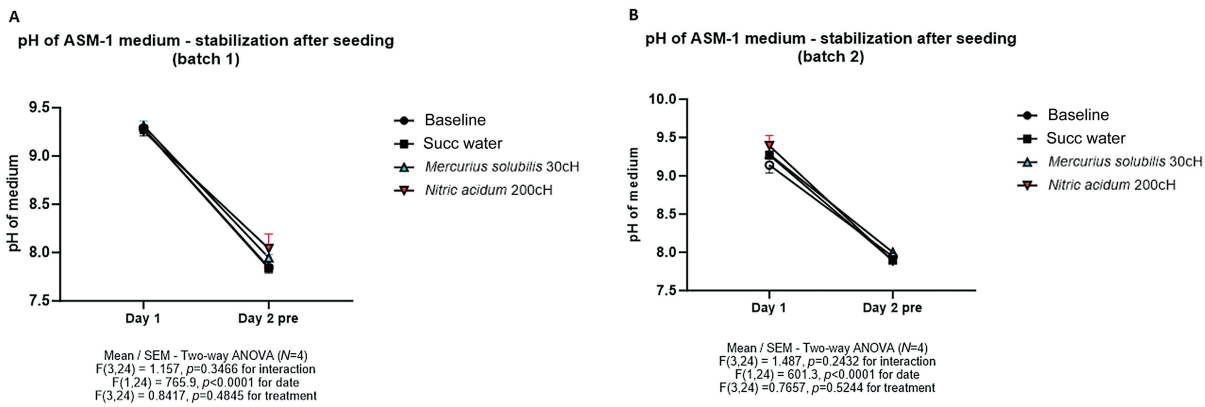

Fig. 7 pH variation of *R. raciborskii* culture medium in the first 24 hours after sowing. (**A**) Batch 1—cultures intended for treatment with homeopathic potencies *not* subjected to a magnetic field. (**B**) Batch 2—cultures intended for treatment with potency subjected to a magnetic field. Two-way ANOVA/Tukey was used since the variables presented Normality in the Shapiro-Wilk test. Statistical data are described at the bottom of the graphic. The mean and standard error represents the data generated in quadruplicates. ANOVA, analysis of variance.

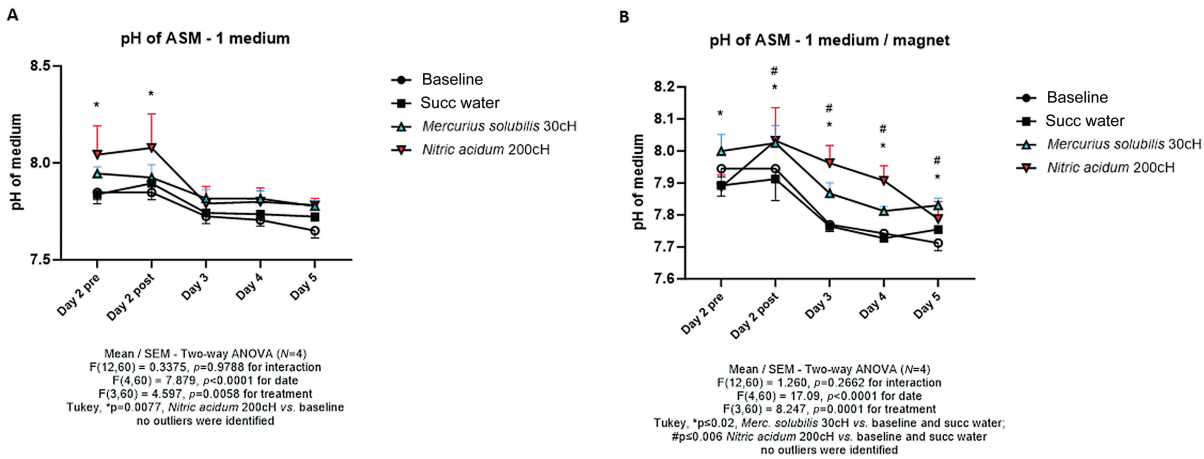

Fig. 8 pH variation of *R. raciborskii* culture medium between days 2 and 5. (**A**) Batch 1—cultures treated with homeopathic potencies *not* subjected to a magnetic field. (**B**) Batch 2—cultures treated with potencies subjected to a magnetic field. Two-way ANOVA/Tukey was used since the variables presented Normality in the Shapiro–Wilk test. Statistical data are described at the bottom of the graphic. The mean and standard error represents the data generated in quadruplicates. ANOVA, analysis of variance.

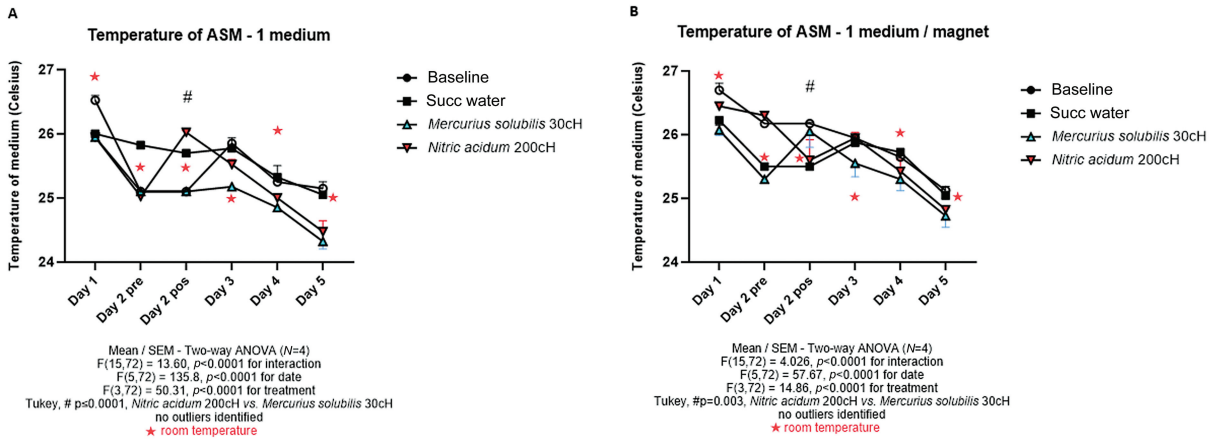

Fig. 9 Temperature variation of *R. raciborskii* culture medium during the experimental period. (**A**) Batch 1—cultures treated with homeopathic potencies *not* subjected to a magnetic field. (**B**) Batch 2—cultures treated with potencies subjected to a magnetic field. Two way-ANOVA/Tukey was used since the variables presented Normality in the Shapiro–Wilk test. Statistical data are described at the bottom of the graphic. The mean and standard error represents the data generated in quadruplicates. The red stars represent the simultaneous ambient temperature. ANOVA, analysis of variance.

(Tukey, $p \leq 0.006$). There was no statistical interaction between time and treatment, revealing a constant interference of homeopathic potencies on the medium culture independent of the time (►**Fig. 8B**).

The medium temperature curve oscillated in relation to the slight variation registered in the laboratory temperature, which was adjusted to 25°C, oscillating from 24.4 to 26.9°C throughout the experimental period. Thus, there was a statistical interaction between time and treatment, independent of submitting the samples to the magnetic field. Interestingly, there was a statistical difference in medium temperature between those samples treated with *Nitric acidum* 200cH and *Mercurius solubilis* 30cH (up to 1°C lower) compared with controls (►**Fig. 9A, 9B**).

Sustained differences are more evident after day 2, and it should be noted that, between days 3 and 5, the pH values of *Nitric acidum 200* cH and M*ercurius solubilis* 30 cH in ASM-1 are consistently higher than control values for both magnet-treated and untreated potencies. Moreover, the higher pH values are mirrored by equally consistent lower temperature values for both magnet-treated and untreated potencies between days 3 and 5.

Discussion

In the present study, we used as a starting point the previous biological results on the effects of different homeopathic preparations on the growth and toxicity of *R. raciborskii* (cyanobacteria), described in the Part 1 paper.[1] Following the mitigation of growth and toxicity observed after the use of certain homeopathic potencies—which could indicate their potential as tools for environmental management of water quality—two crucial questions have arisen: (1) "Is there a correlation between biological effects and physico-chemical changes in water after treatment with homeopathic potencies?"; (2) "Are homeopathic potencies traceable in water media, allowing evaluation of their activities even in natural environments?". The results presented here represent an essential contribution to answering these questions.

The possibility of using homeopathic preparations in natural environmental conditions leads to the need to track their activity when inserted in large bodies of water, as previously demonstrated.[22] There is a need to identify physico-chemical markers that indicate the presence of homeopathic medicines in liquid media. This seems to be possible using solvatochromic dyes.[19,26] Identification of potencies was carried out using a wide selection of dyes that absorb in different ranges of the visible light spectrum and to find those specific dyes that could act as ideal markers for particular potencies.

Considering the possible electromagnetic nature of the interaction between homeopathic potencies and solvatochromic dyes,[23,24] an additional protocol was proposed. Inspired by previous results in this field of study,[27–31] samples were subjected to a known magnetic field before being tested to ascertain if such a procedure enhanced the interaction of potencies with solvatochromic dyes. The exposure time was set as 15 minutes, based on a previous study.[32] However, in this study, the effect of homeopathic potencies' exposure to the magnetic field was measured by means of biological effects, which were canceled after the exposure. Since no study about a magnetic field's influence on other physico-chemical parameters related to homeopathic potencies was found in the literature, this 15 minutes period of exposure was adopted herein to verify if any effect could be observed on solvatochromic dyes' interaction in an exploratory approach of the initial dipole interaction hypothesis. Thus, the variance reduction and the sharper effects of homeopathic potencies on the dyes compared to the controls are findings that corroborate this hypothesis. Other samples of different homeopathic potencies were also tested in this model, equally resulting in sharper results.[33] Specific studies are still needed to verify the reproducibility and generality of the biological effects reported before.[32] Moreover, the existence of discrepant effects of magnetized

potencies on solvatochromic dyes and on biological systems should also be considered.

Results in ►**Figs. 1** and **2** show that Coumarin 7 and Nile red were the best options for identifying the studied potencies, especially after being subjected to a constant strong and unidirectional magnetic flow for 15 minutes. Therefore, using the protocol outlined above, these dyes were chosen to analyze the treated seawater harvested from *Artemia franciscana* cultures and the treated ASM-1 harvested from *R. raciborskii* cultures, whose biological results have been reported.[1] The results indicated the possibility of tracking the activity of Isotherapeutic (*R. raciborskii* extract) 200cH, hydrochloric acid 1cH, *Nitric acidum* 6cH and *Plumbum metallicum* 6cH in seawater previously submitted to a magnetic field using Coumarin 7. On the other hand, Nile red enabled the tracking of *Nitric acidum* 200cH and *Mercurius solubilis* 30cH potencies in ASM-1 after submitting those potencies to a magnetic field.

Using the established protocol, the results strongly suggest that the influence of a magnetic field could be a helpful methodological step when using solvatochromic dyes to track homeopathic potencies inserted in water bodies from different natural settings. From a practical point of view, this discovery is crucial since it might represent a stable tracking process independent of geographical conditions in the natural setting. Although a straightforward tracking process has been seen in a previous study,[22] increasing the method's sensitivity would enable its more general use under different conditions in any part of the globe.

An essential detail of the method used is how samples are prepared before interacting with the dyes, and this has been described and standardized in previous studies.[18,19,22,26] For all samples, regardless of origin (potentized medicines, water, culture media), a further potency (1cH) of each sample is made in sterile pure water or 30% hydroalcoholic solution (according to the protocol), previously filtered in a 0.22-micrometer mesh filter to avoid eventual contamination. Therefore the sample added to dye solutions corresponds to 1% of the original content. By adding the 1cH preparation into the dye solution at a ratio of 1:60, the final concentration of the original content is then 1:6,000.

Samples of seawater and ASM-1 showed more significant variance than samples of pure homeopathic potencies in their interaction with solvatochromic dyes, even after treatment with a magnetic field, which is understandable given the heterogeneity of the starting material and, therefore, potential interference between residual metal ions and the dyes.[20] However, this fact did not prevent the identification of potencies in the respective liquid media, which is crucial considering the intention to use this methodological approach in further studies using water samples from the natural environment, even polluted ones.

Additional physico-chemical parameters (temperature, pH, conductivity) were analyzed directly in ASM-1 containing *R. raciborskii*. No significant differences in conductivity were found between controls and samples (►**Fig. 5**). However, this is perhaps not surprising given the ASM-1 composition, which has high concentrations of salts and metals. Any conductivity changes induced by potencies would be undetectable against a background of such high conductivity. Previous studies on conductivity variations in other homeopathic medicines[34–37] have shown differences, and this physico-chemical parameter is perhaps worth pursuing in future studies.

In contrast, the temperature and pH changes induced by potencies are particularly interesting. For the ASM-1 solutions in ►**Figs. 7** and **8**, changes in pH that follow the addition of both *Nitric acidum* 200cH and *Mercurius solubilis* 30cH inversely correlate with temperature changes. This is the case with and without prior exposure of potencies to a magnetic field. This mirroring is especially clear on days 3 to 5, when it can be assumed that solutions have settled down following mixing since baseline and succussed water controls have similar values. The correlated decrease in temperature and increase in pH relative to controls are likely to be genuine as pH is expected to rise as the temperature falls because pK_a values increase with decreasing temperature, and this is what is seen. If the potency-induced decrease in temperature is genuine (and it appears to occur in all four experiments in ►**Figs. 7** and **8**), this requires an explanation.

The first possible explanation is that the observed effects are somehow associated with *R. raciborskii* growth. However, this explanation is unlikely, as no statistical interactions are seen between bacterial filament numbers and any of the controls or samples as a function of time (►**Supplementary file 5**, available in the online version). Without a biological explanation for the observed sustained increase in pH and associated decrease in temperature (from days 3 to 5, as seen in ►**Figs. 7** and **8**), it becomes necessary to look to a possible chemical explanation. A decrease in temperature indicates an endothermic reaction is taking place in which heat is being taken from the surroundings.

For an endothermic reaction to occur spontaneously,

$$\Delta G = \Delta H - T\Delta S < 0$$

where ΔG is the free energy exchange between the system and surroundings, ΔH refers to the heat change for the reaction, T is the temperature in degrees Kelvin and ΔS is the change in entropy. In other words, if ΔH is positive (which is the case in endothermic reactions), $T\Delta S$ must be strongly negative for the reaction to occur spontaneously. This would then mean the level of order in the surroundings will increase (decreased entropy) and that in the system will decrease (increased entropy).

It would be helpful, therefore, to know what kind of endothermic process could be occurring in a solution that would lead to the system attaining a higher energy level (ΔH positive). Some clues may be forthcoming from a series of studies carried out by Elia et al over several years.[29,38,39] Evidence indicated that dissipative structures were being formed over time in solution by potencies and that these structures produced heat (an exothermic reaction) on alkali treatment. It would seem then that potencies can induce an endothermic reaction to take place in the solution, which involves some structuration, and the reversal of this process (de-structuration) releases heat in an exothermic reaction.

The enthalpy increase ($\Delta H > 0$) in an endothermic process usually results in $\Delta G = \Delta H - T\Delta S > 0$ and so such reactions therefore rarely occur spontaneously. If the system's entropy also decreased (which is what one would expect if structuration were taking place), this would disfavor such a reaction occurring even more as $\Delta G = \Delta H - T\Delta S >> 0$. Such a reaction could only occur if it were driven by some external energy source (e.g., mechanical, electrical or photonic).[40,41] The implication here is that potencies are providing energy to drive a reaction that would not otherwise occur. The nature of this energy and the reaction being driven are open to speculation. Still, it would be unwise to assume that the energy takes any familiar form, such as electrical or photonic. We must entertain the possibility that potencies constitute an utterly unknown form of energy.

Previous studies have shown that pH can be a physico-chemical marker capable of differentiating homeopathic potencies, but in a less specific way than using solvatochromic dyes.[37] In addition, it has been repeatedly noted that potencies appear to raise the pK_a value of many solvatochromic dyes, resulting in increased protonation relative to controls.[21,23–25] However, to our knowledge, temperature changes have not hitherto been investigated in conjunction with changes in pH. From the current study, temperature is likely the parameter of greater importance. Clearly, much more work needs to be done in the area of pH and temperature under purely physico-chemical conditions to verify and extend these findings.

The temperature jumps following the insertion of *Nitric acidum* 200cH and *Mercurius solubilis* 30cH into ASM-1 may also be significant. Yet there is also a possibility that they constitute a mixing artifact, as solutions settle down and become more stable after, or before, 24 hours, as pointed out above. In biological terms, it is known that *R. raciborskii* is quite tolerant to temperature variations.[42–44] Thus, it would be unlikely that the effects of homeopathic potencies on cyanobacteria growth could be attributed to a non-specific effect related to such mild oscillations.

The role of a constant, intense and unidirectional magnetic flow as an amplifying agent in the interaction of the samples with solvatochromic dyes was a significant finding since it could represent an improvement in detection sensitivity. Whether magnetically treated potencies have enhanced biological effects is a subject for future studies. In addition, the effect of varying magnetic field intensity and direction also needs to be investigated in relation to the mechanism of action of homeopathy.

In short, the results obtained in this study indicate the possibility of monitoring the presence of homeopathic medicines in natural water sources and of developing sensitive chemical detection methods as environmental management tools. Given the recent trend of developing new technologies using potentized high dilutions in agriculture, tracking techniques are crucial to their safe inclusion in the production chain, in such a way that observes directives by the FAO (Food and Agriculture Organization of the United Nations) toward the "One Health" approach[45,46] and sustainable development goals for the coming decades.[45]

Conclusion

Tracking homeopathic potencies' activity using solvatochromic dyes has shown to be possible under conditions that mimic those encountered in nature; pH and temperature effects are two additional physico-chemical parameters for tracking, though they are less specific. However, the real value of solvatochromic dyes lies in being able to provide thermodynamic information about the nature of homeopathic potencies, though more studies using this model must be done to confirm it.

The subjection of homeopathic potencies to a constant, intense and unidirectional magnetic flow appears to significantly increase their strength and reduce their variance, as judged by their interaction with solvatochromic dyes. This constitutes an important step in developing a reliable tracking protocol for homeopathic medicines in the wild and furthering our understanding of the fundamental nature of homeopathic potencies.

Highlights

- The putative mitigation of growth and toxicity of *R. raciborskii* with homeopathy was verified—see Part 1.
- The solvatochromic dyes method showed specific traceability for homeopathic potencies in water.
- This effect was more significant when samples were subjected to a static, unidirectional and strong magnetic field.
- Changes in medium pH and temperature indicated endothermic effects after treatment.
- Temperature and pH were less specific for providing potency tracking.

Supplementary Material

Supplementary file 1. Standard solvatochromic dyes.
Supplementary file 2. The neodymium magnet method.
Supplementary file 3. Experimental set-up.
Supplementary file 4. The screening of potencies.
Supplementary file 5. Number of filaments in ASM-1.

Author Attributions

S.N.M. was the main researcher, Ph.D. 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.L.T. was responsible for the discussion of results on physicochemical parameters.
S.J.C. was responsible for experimental design with solvatochromic dyes and the discussion of results.
J.S.Y. was the co-adviser and oversaw cyanobacteria standards and contributed to the discussion about results.

L.V.B. was the main adviser and was responsible for the coordination of all steps of the study and contributed to the discussion about results.

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Conflict of Interest
None declared.

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