UNIVERSIDADE PAULISTA

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ECO-HOMEOPATIA: AS POTÊNCIAS HOMEOPÁTICAS REGULAM O CRESCIMENTO E A TOXICIDADE DE *Raphidiopsis raciborskii* (CIANOBACTÉRIAS) E PODEM SER RASTREADAS FÍSICO-QUIMICAMENTE

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Tese apresentada ao programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista – UNIP, para obtenção do título de Doutor em Patologia Ambiental e Experimental.

Orientadora: Profa. Dra. Leoni Villano Bonamin

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Defesa apresentada ao Programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista – UNIP.

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“Em algum lugar, alguma coisa incrível está esperando para ser descoberta.”

Carl Sagan

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graphic. The data generated in quadruplicates are represented by mean and standard error. The red stars represent the simultaneous ambient temperature.

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RESUMO

Introdução: As cianobactérias são microrganismos encontrados em diversas partes do mundo, sendo vários gêneros produtores de cianotoxinas. Verificou-se que as potências homeopáticas modulam a toxicidade em diferentes modelos biológicos, e o presente estudo procura descobrir se esse também pode ser o caso das cianobactérias. **Objetivos:** a) investigar os efeitos potenciais de potências homeopáticas na resiliência de embriões de *Artemia franciscana* (camarão-de-salmoura) à saxitoxina (cianotoxina) e no crescimento de *Raphidiopsis raciborskii in vitro*; b) verificar a correspondência dos parâmetros físico-químicos das potências específicas com os respectivos efeitos biológicos. **Método:** Cistos de *Artemia franciscana* foram cultivados em água do mar em placas de 96 poços para avaliar a taxa de eclosão, vitalidade e expressão gênica de proteínas de choque térmico (Hsp), após serem desafiados com extrato de *R. raciborskii* contendo 2,5 µg/litro de saxitoxina e tratados com diferentes potências homeopáticas. Culturas de *R. raciborskii* mantidas em meio ASM-1 foram igualmente tratadas com potências homeopáticas, e seu crescimento foi monitorado em função do tempo. As propriedades físico-químicas dos respectivos meios aquosos foram avaliadas por interação com corantes solvatocrômicos e medidas de pH, condutividade e temperatura. A avaliação da expressão gênica de proteínas de choque térmico foi realizada com as amostras de *Artemia franciscana.* **Resultados:** Observou-se menor taxa de eclosão de cistos de *Artemia franciscana* após tratamento com *Nitric acidum* 6cH (p=0,03), e reversão completa da toxicidade com aumento da expressão gênica de Hsp26 e p26 após tratamento com isoterápico (extrato de *R. raciborskii*) 200cH (p≤0,02). O *Nitric acidum* 200cH e *Mercurius solubilis* 30cH limitaram o crescimento exponencial das cianobactérias (p≤0,003) independentemente de sua concentração. A cumarina 7 provou ser um marcador para o *Nitric acidum* 6cH e isoterápico 200cH, mesmo quando inserido na água do mar. O vermelho do Nilo mostrou ser um marcador para o *Nitric acidum* 200cH e *Mercurius solubilis* 30cH, mesmo quando inserido em meio ASM-1 (p≤0,05). Observou-se aumento do pH e efeitos endo/exotérmicos do meio ASM-1 após esses tratamentos (p≤0,007). As análises físico-químicas foram otimizadas após a prévia submissão das amostras a fluxo magnético médio de 2400 Gauss, de forma constante e unidirecional. **Conclusão:** O isoterápico 200cH melhorou a biorresiliência da *Artemia franciscana* à própria

saxitoxina; O *Nitric acidum* 200cH e *Mercurius solubilis* 30cH limitaram o crescimento de *R. raciborskii*. Em todos os casos, as potências homeopáticas que apresentaram efeito biológico foram passíveis de rastreamento no meio aquoso usando corantes solvatocrômicos, em especial quando as amostras foram submetidas ao campo magnético. Os resultados apontam para o potencial da homeopatia na mitigação de problemas ambientais relacionados à qualidade da água.

Palavras-chave: saxitoxinas; *Artemia franciscana*; bioresiliência; água; Hsp; ecotoxicologia.

ABSTRACT

Introduction: Cyanobacteria are microorganisms found in many parts of the world, and several genera are cyanotoxins producers. 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:** a) to investigate potential effects of homeopathic potencies on the resilience of *Artemia franciscana* (brine shrimp) embryos to saxitoxin (cyanotoxin) and on the growth of *Raphidiopsis raciborskii in vitro*; b) to verify any correspondence of physicochemical parameters of specific potencies to respective biological effects. **Method:** *Artemia franciscana* cysts were cultivated in seawater in 96-well plates to evaluate the hatching rate, vitality, and gene expression of heat shock proteins (Hsp), after being challenged with *R. raciborskii* extract containing 2.5 µg/liter of saxitoxin and treated with different homeopathic potencies. Cultures of *R. raciborskii* maintained in ASM-1 medium were equally treated with homeopathic potencies, and their growth was monitored as a function of time. The physicochemical properties of the respective aqueous media were evaluated by interaction with solvatochromic dyes and pH, conductivity, and temperature measurements. **Results:** A lower rate of hatching of *Artemia franciscana* cysts was observed after treatment with *Nitric acidum* 6cH ($p=0.03$), and a complete toxicity reversal with increased Hsp 26 and p26 gene expression was seen after treatment with Isotherapic (*R. raciborskii* extract) 200cH ($p\leq0.02$). *Nitric acidum* 200cH and *Mercurius solubilis* 30cH limited the exponential growth of cyanobacteria ($p\leq0.003$) regardless of their concentration. Coumarin 7 proved to be a marker for *Nitric acidum* 6cH and Isotherapic (*R. raciborskii* extract) 200cH, even when inserted into seawater. Nile red was a marker for *Nitric acidum* 200cH and *Mercurius solubilis* 30cH, even when inserted into ASM-1 medium ($p\leq0.05$). An increase in pH and endo/exothermal effects of the ASM-1 medium was observed after these treatments ($p\leq0.007$). The physicochemical analyses were optimized after submitting the samples to a constant unidirectional 2400 Gauss magnetic flow. **Conclusion:** The isotherapeutic 200cH improved *Artemia franciscana* bioresilience to saxitoxin itself; *Nitric acidum* 200cH and *Mercurius solubilis* 30cH limited the growth of *R. raciborskii*. In all cases, those homeopathic potencies that showed biological effects were traceable in the aqueous medium using solvatochromic dyes, especially when

the samples were subjected to the magnetic field. The results point to the potential of homeopathy in mitigating environmental problems related to water quality.

Keywords: saxitoxins; *Artemia franciscana*; bioresilience; water; Hsp; eco-toxicology.

Capítulo 1

1. INTRODUÇÃO

1.1. Cianobactérias

Cianobactérias são microrganismos procariontes fotossintetizantes que colonizam todos os ecossistemas do planeta, mas são comumente encontrados no plâncton de ambientes marinhos e de água doce. Esses organismos, sob determinadas situações, formam florações do fitoplâncton em corpos d'água. Esse termo é dado quando o número total de células naquele local passa a ser maior que a média do corpo d'água em que aquele microrganismo está presente [1].

Conhecidas como algas azuis, as cianobactérias são microrganismos aeróbicos fotoautotróficos, ou seja, elas obtêm energia a partir de reações químicas do oxigênio (O_2) e a partir de luz solar. Seus principais processos vitais requerem água, dióxido de carbono, substâncias inorgânicas e luz, sendo a fotossíntese seu principal mecanismo de produção de energia [1].

Basicamente, a morfologia das cianobactérias compreende formas unicelulares, coloniais e de filamentos multicelulares, os quais podem conter ou não células especializadas ou diferenciadas, como heterocistos ou acinetos [2].

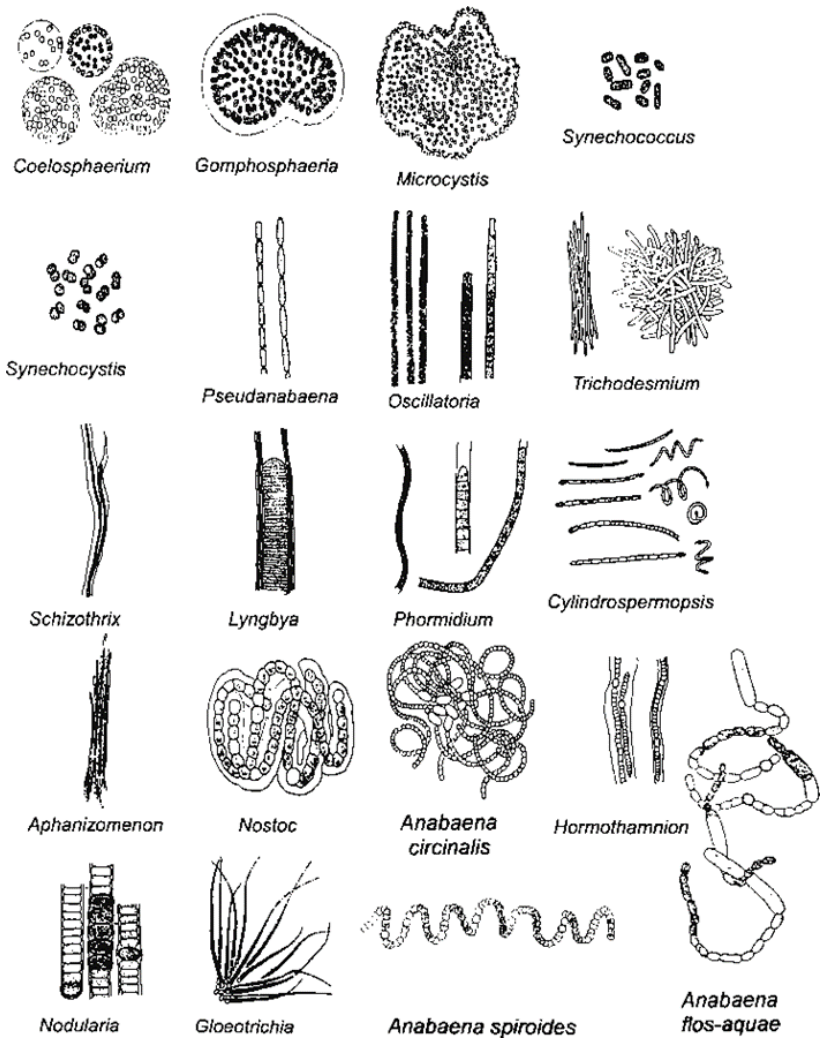
A maioria das espécies planctônicas de cianobactérias tendem a crescer mais em ambientes com água neutro-alcalinas (pH 6-9), com temperatura de 15 a 30ºC e com alta concentração de nutrientes, principalmente nitrogênio e fósforo, ou seja, ambiente eutróficos. Entretanto, uma característica muito importante desses microrganismos é a capacidade de crescer nos mais diversos meios, com predomínio em ambientes límnicos e marinhos, podendo florescer em qualquer tipo de água e durante estações frias e quentes. Além disto, as cianobactérias são capazes de fixar o nitrogênio do ar e formar um estoque para situações em que o nitrogênio dissolvido na água se apresenta escasso [3, 4].

Independente da morfologia e da apresentação celular (coloniais ou filamentosas), alguns gêneros de cianobactérias são capazes de produzir cianotoxinas, como as espécies dos gêneros *Cylindrospermopsis* (*Raphidiopsis*), *Nostoc*, *Oscillatoria*, *Anabaena*, *Aphanizomenon*, *Microcystis* e *Lyngbya*. Existem três

principais tipos de cianotoxinas: neurotoxinas, hepatotoxinas e dermatotoxinas. Essas toxinas são produtos naturais do metabolismo secundário, entretanto, suas funções não estão claramente elucidadas [5,6] (Figura 1).

As cianotoxinas são classificadas conforme sua atividade farmacológica. Os efeitos das neurotoxinas envolvem tontura, adormecimento da boca e extremidades do corpo, taquicardia, fraqueza muscular, náusea e vômitos. Entretanto, principal alvo das hepatotoxinas é o fígado, e essas são promotoras de tumores mediante exposição crônica e até mesmo carcinoma hepatocelular [8], enquanto as dermatotoxinas, em contato com pele e mucosas, causam irritação, podendo levar aos sintomas de vermelhidão, lesões na pele, irritação nos olhos, obstrução nasal e conjuntivite [9].

Figura 1. Gêneros de cianobactérias potencialmente tóxicas.



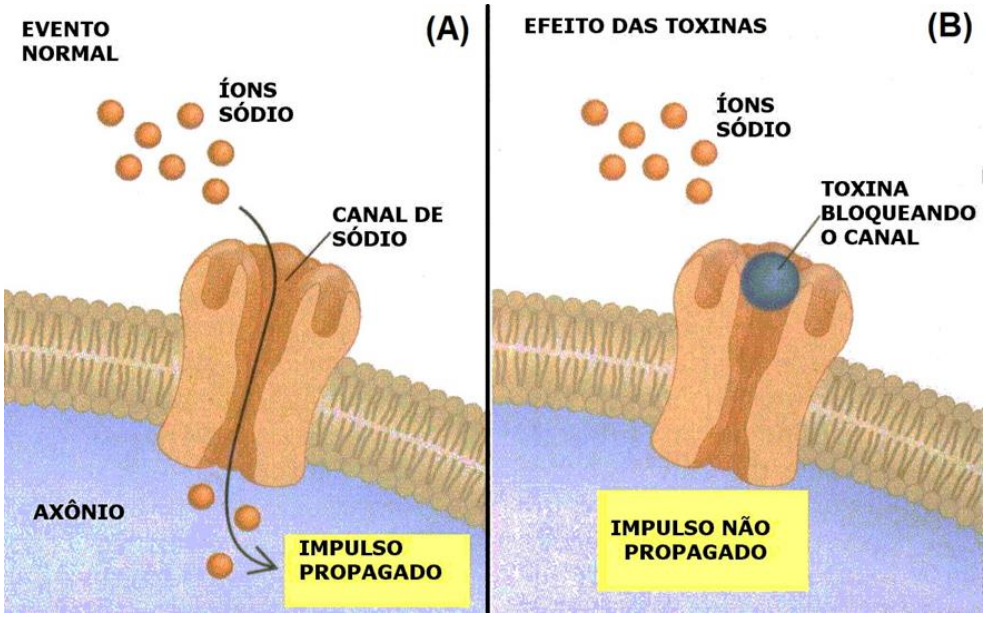
Fonte: adaptado de Lawton *et al.*, 1999 [7].

1.1.1. *Raphidiopsis raciborskii* e as saxitoxinas

Raphidiopsis raciborskii (anteriormente chamada de *Cylindrospermopsis raciborskii*) é uma cianobactéria endêmica no Brasil, produtora de neurotoxinas. Nos demais continentes produz o alcaloide hepatóxico cylindrospermopsina. As *R. raciborskii* brasileiras têm sido documentadas como formadoras de florações, sobretudo em reservatórios e mananciais importantes de abastecimento de água, como os reservatórios Billings, Taiaçupeba e Taquacetuba em São Paulo [10,11].

A cianobactéria *R. raciborskii* é produtora de uma neurotoxina conhecida como a toxina paralisante de marisco (ou *paralytic shellfish toxins* – PTS), cujo nome genérico é saxitoxina. As neurotoxinas são um grupo de alcalóides carbamatos, a toxicidade desse grupo varia bastante, entretanto, a saxitoxina é a mais potente [12,13]. Estima-se que, em uma escala global, cerca de 2.000 casos de intoxicação humana por neurotoxinas são registrados anualmente por consumo de peixes ou mariscos que se alimentaram de algum organismo marinho produtor de saxitoxinas. Desses casos, 15% apresentam mortalidade [14]. Essas neurotoxinas bloqueiam os canais de sódio e cálcio, inibindo a condução nervosa (Figura 2). Um neurônio em repouso tem uma voltagem em sua membrana chamada de potencial de repouso da membrana, esse potencial é determinado pelo gradiente de concentração do sódio (Na+) e potássio (K+). Os canais de sódio, ao estarem bloqueados pelas saxitoxinas, implicam em concentrações alteradas de sódio intracelular, o que afeta a permeabilidade ao potássio e, portanto, a resistência das membranas à condução do impulso, reduzindo seu potencial de ação [15].

Figura 2. (A) Evento normal onde os íons de sódio conseguem atravessar o canal de sódio e propagar o impulso. (B) Efeito das toxinas em que as moléculas de saxitoxina interferem na comunicação entre neurônio e células musculares. Essas moléculas bloqueiam os canais de sódio impedindo a transmissão de impulsos nos nervos periféricos e músculos esqueléticos.



Fonte: adaptado de Carmichael, 1995 [17].

Os sintomas da intoxicação por saxitoxinas podem começar cinco minutos após a ingestão e a morte pode ocorrer entre duas e doze horas. Em caso de intoxicação com doses não letais, os sinais e sintomas desaparecem de um a seis dias. Os efeitos crônicos da intoxicação por saxitoxinas ainda não são claramente elucidados, mas dois aspectos importantes foram documentados: efeitos sobre a memória aversiva em mamíferos e alterações na recuperação de lesões musculares em pacientes acidentados [16].

1.1.2. O problema das cianobactérias e impacto ecológico

Acredita-se que a considerável presença de populações tóxicas cianobactérias nas águas, principalmente de reservatórios, implicam em potenciais danos à saúde da população, uma vez que os mananciais são utilizados para diversos fins, como aquicultura, pesca, uso domiciliar e lazer. Além da exposição à ingestão das cianotoxinas pela população, outra via de contaminação prejudicial é o consumo de organismos aquáticos, uma vez que as cianotoxinas podem se acumular nos músculos de peixes [18].

No Brasil, um trabalho realizado por Teixeira *et al.* (1993) descreve uma forte correlação positiva entre a ocorrência de florações de cianobactérias no reservatório de Itaparica, na Bahia, e a morte de 88 pessoas, entre as 200 que foram relatadas como intoxicadas, através do consumo de água do reservatório entre março e abril de 1988 [19].

Algumas cianotoxinas são bioacumuláveis e podem ser bioamplificadas ao longo da cadeia alimentar, ou seja, alguns animais podem acumular microcistinas e nodularinas (provenientes das cianotoxinas) em sua musculatura. Com isso, outros animais receberão como alimento linhagens tóxicas de cianobactérias. Além disto, as cianobactérias também são responsáveis pela alteração nas populações de peixes, com diversos registros de mortes massivas em resposta ao aparecimento de florações [20].

Não obstante a isso, as cianotoxinas também são deletérias para os animais presentes no meio em que elas se encontram. Essas toxinas não estão disponíveis para os animais apenas quando solúveis, mas também pela ingestão acidental dos seus produtores (cianobactérias), por serem muito pequenos. Um estudo feito por Zagatto *et al.* (2012) em camundongos, microcrustáceos (*Daphnia similis*) e em pulgas d'água (*Ceriodaphnia dubia*) testou os efeitos de dois extratos de *R. raciborskii* sobre esses animais. Como resultado, observou-se efeitos tóxicos em todos eles: os camundongos apresentaram sintomas típicos de neurotoxicidade, como tremores, convulsões e morte, e as outras espécies apresentaram sintomas como imobilização [21, 22].

1.2. Homeopatia e seus principais conceitos

A homeopatia, no contexto terapêutico, emprega o conceito de "cura pela similitude", ou seja: doses infinitesimais de substâncias medicinais são administradas ao paciente cujos sintomas são semelhantes àqueles causados a pessoas sadias pela mesma substância, em um processo denominado "patogenesia". Quando utilizadas em doses extremamente fracionadas e diluídas, submetidas à agitação ritmada, são capazes de manter a informação da substância de origem de alguma forma registrada no solvente para permitir efeitos biologicamente específicos. Sabe-se que os medicamentos homeopáticos podem ser usados tanto em pacientes humanos quanto

em animais (vertebrados ou invertebrados), plantas, células isoladas e microrganismos [23-25].

1.2.1. Princípios da homeopatia

A homeopatia tem como principais pilares o princípio de similitude, a experimentação no indivíduo sadio e o medicamento dinamizado.

1.2.1.1. Princípio da similitude

A homeopatia foi fundamentada pelo médico alemão Samuel Hahnemann, em 1796. Em seus estudos, Hahnemann discorre sobre as propriedades farmacológicas de muitas substâncias medicamentosas utilizadas na época. Com isto, ele conseguiu observar os efeitos primários dos medicamentos, que promovem alterações nos diversos sistemas orgânicos e os efeitos secundários do organismo, que atuariam como resposta neutralizadora aos distúrbios primários [26].

Com base nisso, ao administrar em indivíduos enfermos substâncias que despertaram sintomas semelhantes em indivíduos sadios, o princípio de similitude terapêutica tem como objetivo estimular a reação homeostática curativa contra a enfermidade, induzindo o organismo a reagir contra os seus próprios sintomas, ou seja, qualquer substância capaz de provocar certos sintomas em um indivíduo sadio, é capaz de curar um enfermo que apresente quadro clínico semelhante, utilizando preparações manipuladas adequadamente [27,28].

1.2.1.2. Experimentação no indivíduo sadio

Para se tornar um medicamento homeopático de uso humano, a substância deve ser experimentada em indivíduos sãos, segundo o protocolo de experimentação patogenética, e ter seus efeitos primários (gerais, mentais e físicos) descritos em matérias médicas homeopáticas. A experimentação no indivíduo sadio é o método investigativo para se adquirir o conhecimento das propriedades curativas das substâncias quando empregadas segundo o princípio de cura por similitude [28].

1.2.1.3. Medicamento dinamizado

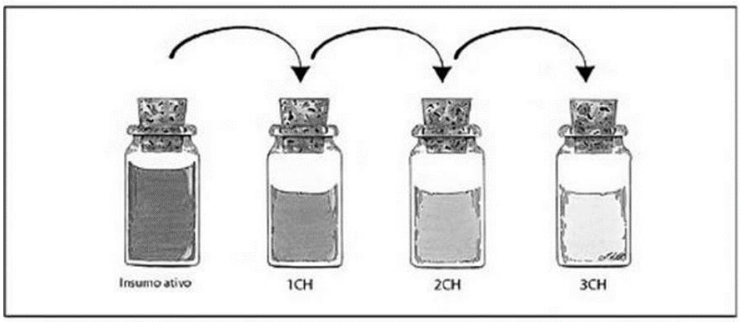
Os medicamentos homeopáticos são substâncias diluídas e agitadas em série. Esse processo é chamado de *dinamização* que, muitas vezes, pode ultrapassar o número de Avogadro (número de átomos por mol de uma determinada substância), ou seja, o limite de 10^{-24} M que representa as soluções em que a probabilidade de existência de

alguma molécula do soluto é zero. Observa-se a ocorrência de atividade biológica destas preparações infinitesimais nos diversos aspectos da individualidade humana e animal, sobretudo nos processos adaptativos aos desafios provenientes do ambiente. A capacidade de “armazenar e transmitir informação” do medicamento homeopático é demonstrada experimentalmente por meio de processos de sinalização entre medicamento e sistema vivo, conforme mostram os estudos de pesquisa básica na área publicados nos últimos 35 anos. Contudo, os mecanismos de ação ainda não estão completamente elucidados [23, 24, 28].

1.2.2. Preparo do medicamento homeopático

Segundo a Farmacopeia Homeopática Brasileira, insumo ativo é “o ponto de partida para a preparação do medicamento homeopático, que se constitui em droga, fármaco, tintura-mãe ou forma farmacêutica derivada”, enquanto insumo inerte é “a substância utilizada como veículo ou excipiente para a preparação dos medicamentos homeopáticos” [29]. Para preparar um medicamento homeopático adiciona-se uma parte do insumo ativo e 99 partes do insumo inerte, que é submetido à sucussão, ou seja: à agitação vertical e ritmada do medicamento, compondo assim a primeira dinamização centesimal Hahnemanniana, ou 1CH. Para se obter a segunda dinamização centesimal Hahnemanniana, 2CH, utiliza-se uma parte da 1CH mais 99 partes do insumo inerte e posterior sucussão. Cada estágio é chamado de “potência homeopática’. A obtenção das demais potências segue o mesmo processo (Figura 3) [29-31].

Figura 3. Preparo do medicamento homeopático, iniciando a partir do insumo ativo, para obtenção da primeira potência (1CH) e das demais potências, sucessivamente.



Fonte: Fontes, 2019 (31).

1.2.3. Homeopatia e isoterapia

Embora todo isoterápico seja um medicamento homeopático por ser produzido a partir dos processos que envolvem a produção de um medicamento homeopático, a diferença principal entre ambas as terapias consiste na metodologia empregada, ou seja, só saberemos se um medicamento homeopático é um isoterápico por meio do contexto terapêutico no qual este medicamento foi inserido. Isto acontece porque na isoterapia, o princípio utilizado, diferentemente da homeopatia, é a igualdade, enquanto na homeopatia, o princípio é por similitude.

A isoterapia tem como objetivo tratar o indivíduo com o mesmo agente etiológico causador da doença, e não com substância capaz de gerar sintomas semelhantes à doença apresentada. Em ambos os casos, o que muda é o insumo ativo utilizado e a circunstância clínica [33]. Por exemplo, em um estudo realizado por Mohammad (2020), utilizou-se o modelo de *Artemia franciscana* para observar os efeitos de três potências de isoterápico (6CH, 30CH, 200CH) cujo insumo ativo era o cloreto de chumbo. Para essa avaliação, os animais foram submetidos à intoxicação por cloreto de chumbo para então receberem o medicamento. Desta forma, todos os animais foram tratados a partir do princípio de igualdade [34].

Na isoterapia, a origem do insumo ativo pode ser endógena ou exógena, podendo ser classificados como autoisoterápicos, em que o insumo ativo é oriundo do próprio paciente e só é destinado a ele, ou heteroisoterápicos, cujo insumo ativo é externo ao paciente enxógeno mas o sensibiliza quando da administração [35].

1.3. *Artemia franciscana* e HSPs

A *Artemia franciscana* (ou artêmia) é um microcrustáceo da ordem *Anostraca* encontrado principalmente em águas salgadas. Esse animal se alimenta basicamente de bactérias, algas unicelulares, pequenos protozoários e detritos dissolvidos no meio [36,37].

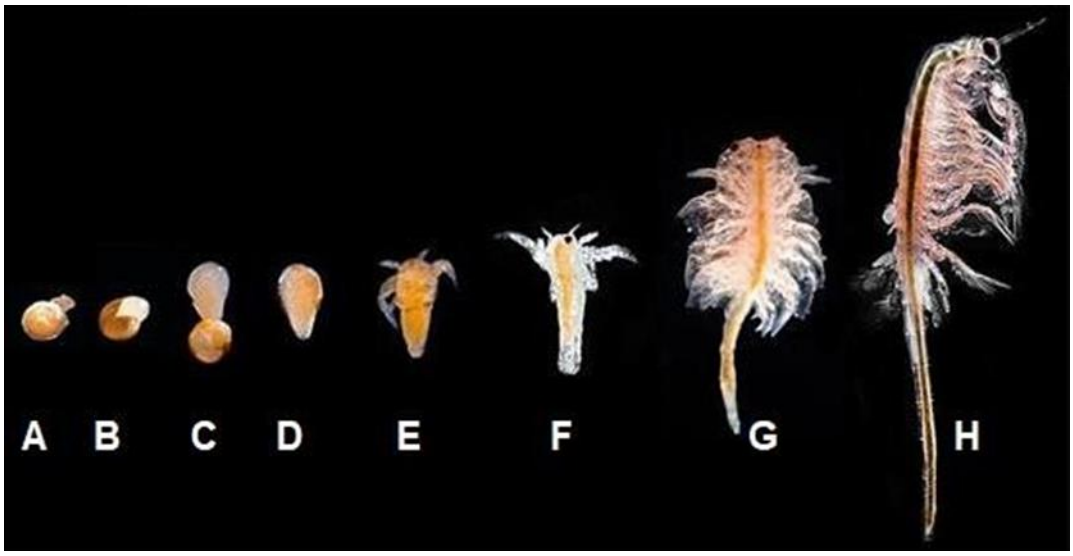
A artêmia tem sido utilizada em testes de toxicidade por sua capacidade de fazer com que seus cistos permaneçam em diapausa, estado em que o metabolismo dos cistos permanece suprimido e há maior tolerância a qualquer fator estressante do meio externo. Além disto, elas são fáceis de manusear e cultivar, é um método barato e relativamente rápido e podem acumular substâncias em seus tecidos [37-39].

Os organismos do gênero *Artemia* são seres extremamente bioresilientes, ou seja, são capazes de se adaptar facilmente às mudanças ambientais. Essa bioresiliência é oriunda da capacidade dos embriões permanecerem em diapausa em seus cistos quando da presença de estímulo nocivo, mantendo-se viáveis. Em situação laboratorial, a menor taxa de eclosão e maior vitalidade dos náuplios é um indicativo de adaptação e bioresiliência desses animais [38-39].

Esse microcrustáceo apresenta diversas fases de desenvolvimento que compreendem: cisto, estágio “guarda-chuva”, náuplio (estágios I a V), juvenil e adulta. Na fase de cisto que pode durar até 25 anos as principais características estruturais se diferem em pré e pós hidratação, visto que na pré-hidratação os cistos apresentam-se como côncavos e na pós-hidratação os cistos estão esféricos. A fase “guarda-chuva” consiste no primeiro estágio pós eclosão, em que o embrião presente no cisto rompe as membranas do mesmo e começa a nadar no meio externo. Essa fase dura de 1 a 8 horas e o animal apresenta pouca atividade muscular. A fase naupliar da *Artemia franciscana* pode ser dividida em 5 estágios instar (I – V) e pode durar até 48 horas; é nessa fase que o animal encontra-se em estágio de larva, inicialmente se alimentando de nutrientes vindos das reservas embrionárias e, posteriormente, por filtração da água. A fase naupliar existe até o animal atingir a fase juvenil e, posteriormente, a fase adulta que pode durar de 8 a 15 dias [39,40] (Figura 4).

Existem alguns estudos que utilizam a *Artemia spp* para testes toxicológicos envolvendo cianobactérias. Um estudo feito por Sirvec *et al.*, em 2016, buscou entender a morte massiva de diversos peixes no lago Aleksandrovac, na Sérvia, que ocorreu durante a floração de *R. raciborski*. Utilizando como modelo experimental a *Artemia franciscana*, foi observada a presença de compostos tóxicos nas cianobactérias [41]. Outro estudo, desta vez realizado por Martin *et al.,* em 2007, também mostrou os efeitos tóxicos de alguns extratos de cianobactérias em *Artemia franciscana* [42].

Figura 4. Diferentes estágios da *Artemia franciscana*, desde a fase de cisto até a fase adulta. (A) cisto hidratado, (B) cisto em ruptura, (C) estágio guarda-chuva, (D, E, F) fase naupliar, (G) fase juvenil, (H) fase adulta.



Fonte: adaptado de Harzsch *et al.*, 2002 [40].

Recentemente, extratos naturais de uma floração da cianobactéria marinha *Trichodesmium*, produtoras de saxitoxinas, provaram ser letais (40% em 48h) em bioensaios com artêmia [43]. Assim, *Artemia spp*, por ser um potencial bioacumulador na cadeia alimentar marinha, pode transmitir neurotoxinas para as demais camadas tróficas marinhas.

As proteínas de choque térmico (*Heat shock proteins* – Hsps) são chaperonas moleculares essenciais para a manutenção das funções celulares, pois possuem a capacidade de interagir com outras proteínas auxiliando na formação, dobramento e transporte das mesmas, além de desempenharem um papel importante na resposta de defesa celular a diversos estímulos estressantes ambientais [48-50].

Recentemente algumas dessas proteínas, como a Hsp70 e Hsp90, foram relatadas desempenhando papéis importantes na apresentação de antígenos, ativação de linfócitos e macrófagos e ativação e maturação de células dendríticas (APCs), sugerindo assim, uma ligação entre as Hsps com o sistema imunológico imune e adaptativo de vertebrados [51-55]. As Hsp70 compõem uma família de proteínas comumente associadas ao início e duração da tolerância à temperatura. As

Hsp90 são necessárias para a estabilidade de várias proteínas de sinalização, mesmo ativadas e/ou expressas [56, 57].

A *Artemia franciscana*, por sua vez, possui características moleculares para sobreviver a condições estressantes, como a proteína de choque térmico p26 (Hsp26) [58, 59]. A Hsp26 é uma chaperona molecular regulada por temperatura capaz de formar grandes complexos oligoméricos. Alguns ensaios realizados em diferentes temperaturas mostram que a dissociação do complexo Hsp26 no choque térmico é um pré-requisito para a atividade eficiente dessas chaperonas. Acredita-se que a maior expressão de Hsp26 e p26 seja um dos mecanismos capazes de fazer com que embriões de *Artemia franciscana* em diapausa se adaptem a ambientes nocivos e permaneçam em condições vitais. [60, 61].

Existem alguns estudos que medem a expressão de proteínas de choque térmico após o uso de terapia homeopática. Um estudo feito por Mukherjee *et al.*, por exemplo, observou se a Thuja 30cH poderia de alguma forma reduzir os efeitos danosos no DNA causado pelo benzopireno. Nesse estudo pôde-se observar que houve menor expressão de Hsp90 [62].

Estudos de termotolerância são muito relevantes para as cianobactérias aquáticas, levando em consideração que seu florescimento está intimamente associado a temperaturas elevadas [63]. Além disto, cianobactérias expressam HtpG e a mutação desse gene pode prejudicar o crescimento e/ou sobrevida delas [64].

2. JUSTIFICATIVA

Os efeitos de cianobactérias produtoras de cianotoxinas é bem conhecido na literatura. Os sintomas dessas cianobactérias variam conforme o tipo de cianotoxina produzida e pode envolver sinais hepáticos, dermatológicos e neuronais, tanto em animais como em humanos. Dependendo do grau de intoxicação, o indivíduo pode vir a óbito. Nos casos menos críticos, a presença de cianobactérias pode implicar em meu odor e sabor indesejável da água a ser consumida.

Em 2020, uma iniciativa realizada pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) em conjunto com a Companhia de Saneamento Básico do Estado de São Paulo (SABESP) propôs encontrar soluções por meio de

projetos de pesquisa cujo objetivo seria controlar a crescente população de cianobactérias nos reservatórios do estado de São Paulo. O conhecimento dessa demanda serviu de motivação para o planejamento e execução desta pesquisa.

O laboratório de Cianobactérias e Ficotoxinas da UFRG tem atuado desde 2019 não só na produção de conhecimento científico, mas também no fortalecimento da visão empreendedora de estudantes na busca de novos fármacos que possam resolver problemas ambientais e sustentabilidade em reservatórios de abastecimento de águas no Brasil. O Prof. João Sarkis Yunes, coordenador do laboratório de cianobactérias da UFRG, aceitou participar do projeto como co-orientador e fornecer as cepas de *R. raciborski*.

Paralelamente, os estudos realizados previamente pelo nosso grupo para a identificação de efeitos de preparações homeopáticas na facilitação dos processos de bioresiliência, utilizando o modelo de *Artemia franciscana* exposta a glifosato e a metais pesados, mostraram efeitos protetores significativos, o que também serviu de *background* para a definição do desenho experimental utilizado.

Por fim, a constatação prévia de que a sinalização causada pela imersão de preparações homeopáticas em grandes volumes de água pode ser rastreada por meio da análise de amostras de água pelo método dos corantes solvatocrômicos, também abriu uma perspectiva prática importante. Sendo assim, uma parte da pesquisa foi dedicada a essa questão.

Com base nesse contexto, o presente estudo tem como principal justificativa buscar soluções baratas e eficazes para o grande problema de crescimento de cianobactérias em mananciais, em uma perspectiva de médio a longo prazo face aos resultados obtidos em laboratório, conforme descritos a seguir.

3. OBJETIVOS

3.1. Objetivos gerais

Esse projeto tem como objetivo observar possíveis efeitos atenuadores de preparações homeopáticas e isoterápicas sobre a toxicidade de extratos de *C. raciborski* em modelo biológico (*Artemia franciscana)*, bem como sobre o crescimento das próprias cianobactérias. Além disto, o projeto busca soluções sustentáveis, baratas e eficazes para o grande problema de crescimento de cianobactérias em

mananciais, utilizando insumos homeopáticos como possíveis instrumentos para tratamento da água.

3.2. Objetivos específicos

1) Determinar a toxicidade de *Raphidiopsis raciborskii* em *A. franciscana* e observar se produtos homeopáticos afetam sua toxicidade por meio de triagem de vários insumos;
2) Observar se produtos homeopáticos influenciam o crescimento dessa cianobactéria por meio de triagem de vários insumos;
3) Observar quais parâmetros físico-químicos são úteis como forma de rastrear a presença de potências homeopáticas na água (água do mar ou meio ASM-1), bem como estabelecer um paralelo com os respectivos efeitos biológicos.

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Capítulo 2

Manuscrito a ser submetido ao periódico *Homeopathy*

O manuscrito apresentado a seguir representa a versão preliminar a ser enviada ao periódico *Homeopathy* (Thieme Publishers, IF = 1.8, Qualis A2), sendo escrito na língua inglesa revisada por coautor nativo, respeitando as normativas de redação exigidas pelo periódico.

Title Page

Eco-homeopathy: Homeopathic potencies regulate the growth and toxicity of *Raphidiopsis raciborskii* (cyanobacteria) and can be tracked by physicochemically

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João S Yunes – Co-advisor, cyanobacteria standards, discussion of results

Leoni V Bonamin – Main Advisor, coordination of all steps of the study, discussion of results

Eco-homeopathy: Homeopathic potencies regulate the growth and toxicity of *Raphidiopsis raciborskii* (cyanobacteria) and can be tracked physicochemically

Abstract

Introduction: Cyanobacteria are microorganisms found in many parts of the world, and several genera are cyanotoxins producers. 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:** a) to investigate possible effects of homeopathic potencies on the resilience of *Artemia franciscana* (brine shrimp) embryos to saxitoxin (cyanotoxin) and on controlling the growth of *Raphidiopsis raciborskii in vitro*; b) to verify which physicochemical parameters can be used to track homeopathic potencies in seawater or ASM-1 medium and establish a parallel with their biological effects**.** **Method:** *Artemia franciscana* cysts were cultivated in seawater in 96-well plates to evaluate the hatching rate, vitality, and gene expression of heat shock proteins (Hsp), after being challenged with *R. raciborskii* extract containing 2.5 µg/liter of saxitoxin and treated with different homeopathic potencies chosen from a screening process. Cultures of *R. raciborskii* maintained in ASM-1 medium were equally treated with previously screened homeopathic potencies, and their growth was monitored as a function of time. The physicochemical properties of treated water were evaluated by the interaction with solvatochromic dyes, and measuring pH, conductivity, and temperature. **Results:** A lower rate of hatching of *Artemia franciscana* cysts was observed after treatment with *Nitric acidum* 6cH ($p=0.03$), and a complete toxicity reversal with increased Hsp 26 and p26 gene expression was seen after treatment with Isotherapic (*R. raciborskii* extract) 200cH ($p\leq0.02$). *Nitric acidum* 200cH and *Mercurius solubilis* 30cH limited the exponential growth of cyanobacteria ($p\leq0.003$) regardless of their concentration. Coumarin 7 was a marker for *Nitric acidum* 6cH and Isotherapic (*R. raciborskii* extract) 200cH, even when inserted into seawater. Nile red was a marker for *Nitric*

acidum 200cH and *Mercurius solubilis* 30cH, even when inserted into ASM-1 medium ($p \leq 0.05$). An increase in pH and endo/exothermal effects of the ASM-1 medium was observed after these treatments ($p \leq 0.007$). The physicochemical analyzes were optimized after the previous submission of the samples to a constant unidirectional 2400 Gauss magnetic flow. **Conclusion:** The Isotherapic 200cH improved *Artemia franciscana* bioresilience to saxitoxin itself; *Nitric acidum* 200cH and *Mercurius solubilis* 30cH limited the growth of *R. raciborskii*. In all cases, those homeopathic potencies that showed biological effects were traceable in the aqueous medium using solvatochromic dyes, especially when the samples were subjected to the magnetic field. The results point to the potential of homeopathy in mitigating environmental problems related to water quality.

Keywords: saxitoxins; *Artemia franciscana*; bioresilience; water; Hsp; eco-toxicology

1. Introduction

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

Raphidiopsis raciborskii (formerly *Cylindrospermopsis raciborskii*) is a cyanobacteria that produces neurotoxins, named saxitoxins. It multiplies in freshwater rich in metals and organic matter, a clear indicator of water pollution.

They form blooms in large water reservoirs and springs [1-4]. Saxitoxins produced by the *R. raciborskii* block sodium and calcium channels, inhibiting nerve conduction, and affecting potassium permeability. 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 *Raphidiopsis raciborskii* extracts are recognized by neurotoxicity signs, such as tremors and convulsions (in mice) and immobilization (in microcrustaceans) [6]. Vilar and 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 saxitoxin intoxication have still not been clearly elucidated. However, two important consequences have been recently documented: adverse effects on aversive memory and delayed recovery from muscle injuries in mammals [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 their muscles [8].

The microcrustacean *Artemia spp* (brine shrimp) is commonly used as an experimental model for toxicity tests [9,10]. 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 (Hsp) 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 the diapause termination [16, 17].

Some studies use *Artemia franciscana* for toxicological tests involving cyanobacteria. A study by Sirvec *et al.* in 2016 sought to understand the massive death of several fish in Lake Aleksandrovac, Serbia, which occurred during the flowering of *R. raciborskii*. Using *Artemia franciscana* as an experimental model, the presence of toxic compounds in water and cyanobacterial cells was identified [18]. Natural extracts from a bloom of marine cyanobacterium *Trichodesmium*, another saxitoxin producer, proved to be lethal in bioassays with the genus Artemia [19]. Thus, *Artemia spp*, as a potential bioaccumulator in the marine food chain, can be considered a good model for identifying cyanotoxins [20]. Also, *Artemia spp.* are deemed to be non-sentient

117 organisms, which significantly facilitates the ethical aspects involving their use in
118 research [10, 21].

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

127 Given that such homeopathic dilutions often exceed the limit of Avogadro's
128 number, it is necessary to consider the possible existence of mechanisms other than
129 purely biochemical ones to explain the observed protective effects described above.
130 From a biological point of view, what appears to be an increase in adaptive processes
131 (or “hormesis”) has been frequently observed in living systems treated with
132 homeopathic dilutions under a range of different circumstances [29-31]. Furthermore,
133 such biological effects have shown close correspondence with physicochemical
134 changes in previous studies using microcrustaceans [22]. These physicochemical
135 changes can be monitored through interactions with solvatochromic dyes [24, 34],
136 based on the method developed by Cartwright [32- 38]. Correspondence between
137 biological effects and physicochemical changes has also been observed in other
138 situations, in both laboratory [39] and natural [35] conditions.

139 Solvatochromic dyes have been found to be probes able to track homeopathic
140 potencies [32-38]. The nature of the interaction between solvatochromic dyes and

141 homeopathic potencies is yet to be elucidated, but the evidence so far indicates that
142 the unique electronic structure and behavior of these dyes is crucial to the interaction
143 and that the interaction involves an increase in their polarity, which can be measured
144 spectroscopically in the UV-visible region of the electromagnetic spectrum.

145 The observation of increased polarity in relation to high dilution activity has been
146 reported since the 1980's [40-44], and it is against a background of these reports that
147 the present study sought to test the hypothesis that homeopathic preparations could
148 be traceable agents in controlling the growth and toxicity of cyanobacteria. The present
149 study is justified by the need to look for cheap and effective homeopathy-based
150 technologies that focus on a common worldwide environmental problem: excessive
151 cyanobacterial growth in water sources.

152

153 **2. Materials and methods**

154

155 The study design was organized as follows:

156 1) Evaluation of *R. raciborskii* toxicity on *A. franciscana* and observation of which
157 homeopathic potencies would be able to mitigate it, by means of cyst-hatching
158 bio-resilience mode and heat-shock proteins gene expression.
159 2) Evaluation of *R. raciborskii* growth rate after treatment with homeopathic
160 potencies poured into the culture medium.
161 3) Evaluation of physicochemical parameters able to be used to track the presence
162 of homeopathic potencies in water medium and establish a parallel with their
163 biological effects.

In all cases, the homeopathic potencies were chosen from a standard screening process, according to the case.

2.1. *R. raciborskii* extract toxicity 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 prepared in hydrochloride acid 0.05M [2].

The toxicity of different batches of T3 extract was evaluated in a preliminary test on the *A. franciscana* cyst (Maramar-pet, Arraial do Cabo, Brazil) hatching rate 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 following 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 magnifying pen-type microscope - 1000x zoom, camera 2.0 megapixels, USB, 6 LEDs (Digital Microscope, Beijing, China), coupled to a computer (Yoga 520, Lenovo, Brazil).

In the first preliminary assay, *Artemia 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 5 to 8 cysts for each fraction of 100µl, to be inserted into each well. Rows of 5 wells were completed for 250µl of seawater containing 1% T3 extract from each batch. Untreated wells (baseline) and 1% hydrochloride acid 0.05M were used as controls. This proportion was chosen after a pilot study to identify the hydrochloride 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.

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 saxitoxin (STX) equivalent concentration, as previously calculated by comparison to a set of SXT standards variants purchased from the NRC-Canada, and analyzed by HPLC-FD methods as described in [45-47]. The known amount of saxitoxin per liter of each extract is described in Supplementary Material 1.

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 were completed for 250µl of seawater containing T3 extract in different concentrations. Untreated wells (baseline) and 1% hydrochloride acid 0.05M were used as controls. After 48 hours of challenging, 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.

To avoid eventual interference of electromagnetic environmental fluctuations face to the known sensitivity of this species [22, 23], all assays involving *Artemia franciscana* were performed in a Faraday cage with 300 micro-led bulbs inside (Supplementary Material 2), allowing a constant low magnetic field (0.06 µT at 50Hz, Smart-Sensor Intel Instruments, AS 1392, Singapore) that is crucial for the homeopathic potency activity [48], and enough light to induce cysts hatching during incubation. Considering previous studies, the same lunar cycle phase was standardized in all experimental series [22, 23], now establishing the crescent moon as the standard.

2.2. Screening of homeopathic potencies for protection of *Artemia franciscana* from *R. raciborskii* extract toxicity

Besides the isotherapeutics (homeopathic dilutions prepared from *R. racibosrskii* extract 5), additional homeopathic preparations were chosen to be tested in a preliminary screening assay, based on the similarity of symptoms between the respective *materia medica* and the symptoms caused by the saxitoxin in humans. A group of three veterinarians and one physician reached the list of medicines by consensus. For each homeopathic medicine, 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].

In this first screening study, the medicines tested 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
- Isoterapeutics prepared from *R. raciborskii* extract 5 - 6cH, 30cH, 200cH

And the controls were:

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

Stock potencies were prepared in a ANVISA (National Agency for Sanitary Surveillance) registered homeopathic pharmacy in São Paulo. They were supplied at one potency level before the working dilution, that is 5cH, 29cH, and 199cH, using 10% alcohol as a solvent. The working potencies were prepared one 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's cultures were made in microplates, as described in item 2.1. 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 in [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, being 2.5 µg/liter the final saxitoxin concentration. Samples were analyzed in sextuplicate; the sum of cysts from each row of wells was considered the experimental unit, although the total number of evaluated cysts was 6594.

The results of this homeopathy screening test were evaluated using a 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:

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

The three selected homeopathic preparations were compared to one another 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^{o}C for further gene expression and physicochemical analyses.

2.3. 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 Heat Shock Proteins (HSPs), reported as key elements in controlling cyst hatching, and the embryo development of *Artemia spp* [11-15] was also investigated.

Gene expression tests were made from the stored frozen biological samples in the screening experiments. In the first step, total RNA extractions were carried out by using TRIzol®/Chloroform/Isopropanol method [49]. For this, the *Artemia 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 0.5ml of TRIzol® (MERCK, Whitehouse Station NJ, USA) and mixed with 0.2ml of chloroform (MERCK, Whitehouse Station NJ, USA). Then, it was centrifuged at 4^{o}C, 14000 rpm, for 15 minutes (Eppendorf 5804R centrifuge, Hamburg, Germany), the water fraction was removed, and the RNA fraction was precipitated by adding 0.5ml of absolute cold isopropanol (MERCK, Whitehouse Station, NJ, USA) for 10ml, at room temperature. Next, the samples were centrifuged again; the RNA pellet was washed in 75% ethanol and suspended in 0.02ml of Diethyl Pyro carbonate (DEPC) aqueous solution. The quality and pureness of the extracted RNA were estimated by spectrophotometry (NanoDrop 2000, Thermo Scientific, USA). 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^{o}C until the HSPs (heat shock proteins) expression assays began.

For gene expression analyses of specific mRNA (*Hsp 40*, *Hsp 90, Hsp 26*, *p26*) [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 manufacturer's instructions. 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), 60 ng of cDNA nuclease-free H_2O in a *QuantStudio® 3* Real-Time PCR (Thermo Fisher Scientific, Waltham, Massachusetts, USA). 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 primers sequence (EXXTEND, Paulinia, Brazil) and PCR conditions are expressed in Table 1.

Table 1. Available *primers* for identifying *Artemia franciscana* Hsps, considered bioresilience markers. Primers were used in the RNA expression assay (qPCR).

Gene	Primer	5'- 3' Sequence	Reactions Condicition
ART_*Hsp26*	Forward Reverse	CGG AGG ATT TGG TGG TAT GAC CCT CAA GGA CCC AGG AGT AG	95°C - 15s; 58°C - 30s; 72°C - 30s
ART_*Hsp40*	Forward Reverse	GTG CAT CAG TTG AGC GTC AC TGCTGAACCATTCCAGGAGC	95°C - 15s; 59°C - 30s; 72°C - 30s
ART_*Hsp70*	Forward Reverse	CGA TAA AGG CCG TCT CTC CA CAG CTT CAG GTA ACT TGT CCT TG	95°C - 15s; 58°C - 30s; 72°C - 30s
ART_*Hsp90*	Forward Reverse	GGT GTG GGT TTC TAT TCT GC GCA GCA GAT TCC CAC ACA	95°C - 15s; 59°C - 30s; 72°C - 30s
ART_*p26*	Forward Reverse	GCG CGG ATC CAC CAT GGC ACT TAA CCC ATG CGC GCC TCG AGT TAA GCT GCA CCT CCT GTC T	95°C - 15s; 57°C - 30s; 72°C - 30s
ART_*At*	Forward Reverse	GCA GTG GTC TAC AAG GTT TC ATC AAA ACG AAG GCT GGC GGT G	95°C - 15s; 60°C - 30s; 72°C - 30s
ART_*Ef1α*	Forward Reverse	TCG ACA AGA GAA CCA TTG AAA A ACG CTC AGC TTT AAG TTT GTC C	95°C - 15s; 60°C - 30s; 72°C - 30s
ART_α-tubulin	Forward Reverse	CTG CAT GCT GTA CAG AGG AGA TGT CTC CTT CAA GAG AGT CCA TGC CAA	95°C - 15s; 60°C - 30s; 72°C - 30s

2.4. *R. 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

329 results were chosen for a complete statistical analysis and further experimental
330 repetitions to confirm the results.

331 The cyanobacteria *R. raciborskii* T3 strain was donated to our laboratory by JSY
332 (LCF-FURG, Brazil). Culture samples were sent to the UNIP Research Center, São
333 Paulo, Brazil, in sealed sterile tubes at room temperature. The cultures were replicated
334 in the standard ASM-1 medium [51] and kept in a separate room at constant
335 temperature (25 ± 1^{o}C) and light cycle (14h light – 10h dark), provided by cold white
336 fluorescent light fixed in the roof. Both environmental parameters were automatically
337 controlled during the whole experimental period. Cultures were kept in 500ml
338 Erlenmeyer containers covered with hydrophobic cotton/gauze cushion and put on a
339 high but open shelf to avoid accidental moving. The shelf had lateral limits protected
340 by wood walls with enough space at the top for ventilation.

341 The cultures were replicated monthly for maintenance, and the experiments
342 were performed after 15 days after a new replication. When the cultures reached the
343 exponential growth phase, they showed typical yellowish color (Supplementary
344 material 3) and filamentous microscopic structures.

345 A screening assay was done to select the best treatments, using a 15-day
346 culture as a starting point. Aliquots of 3 ml containing 5 x 10^{7} filaments/liter were
347 distributed in 69 culture tubes (20ml, transparent glass) covered with hydrophobic
348 cotton but kept with an air column to allow enough oxygenation (Supplementary
349 Material 3). Each treatment was performed in triplicate.

350 This bacterial concentration (5 x 10^{7} filaments/liter) corresponds to the amount
351 needed to produce 3.6 µg/liter of saxitoxin, according to the standards previously
352 defined [45], by using the formula:

y = 0.073x -1.5065

being

y = saxitoxin concentration (µg/liter) and x = number of filaments/ml

This saxitoxin concentration is close to the maximum limit allowed in reservoirs by the Brazilian Health Ministry (*MS* 2914/2011), which is 3.0µg equivalent saxitoxin/liter [53].

The counting of filaments was performed three times a week for three 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 = 2500y

being

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

For the treatments, a volume of 0.3 ml (3% 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 in item 2.1. Baseline (no treated cyanobacteria), autoclaved pure water, and autoclaved succussed pure water were used as controls, prepared as described in item 2.1. All cyanobacteria manipulation was done in a laminar flow cabin to prevent contamination.

In the first screening, the twenty-two homeopathic potencies were tested for hatching rate and nauplii vitality (live-born nauplii/cysts number ratio). 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.

The treatments were randomly numbered by a person not involved in the experiment. All experiments were done blind, 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
- Isoterapeutics prepared from *R. raciborskii* extract 5 - 6cH, 30cH, 200cH

And the controls were:

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

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

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

And the controls were:

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

The main experimental set was done using the same protocol described for the screening phase, being N=9, that is, nine tubes for each chosen treatment or control.

In this case, the initial number of filaments was 20 x 10^7 per liter, corresponding to 14.6 µg/liter of saxitoxin, to challenge the effectiveness of treatments in the worst conditions, with a significant population of *R. raciborskii.*

2.5. Sample analysis by solvatochromic dyes method

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. Different solvatochromic dyes respond to homeopathic potencies according to their structure, so selective detection and identification of different homeopathic potencies may be possible. The present study aims, amongst others, to see if this possibility can be realized. [32-38].

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

2.5.1. Sample preparation

All samples followed a previously standardized preparation protocol [39]. In laminar flow, 10ml 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, another 100µl of each previously filtered sample

(Merck–Millipore, Darmstadt, Germany) was added. Flasks were closed and submitted to 100 succussions in the automatic robotic arm (Denise-Autic, São Paulo, Brazil).

Each medicine sample was prepared from the respective stock potency. Each water sample (ASM-1 medium or seawater) was prepared from the liquid content of a pool of wells submitted to the same treatment 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 were:

- Water (baseline)
- Succussed autoclaved pure water (treatment control)
- Hydrochloric acid 1cH (5 x 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 were those treated with:

- *Plumbum metallicum* 6cH
- *Nitric acidum* 6cH
- Hydrochloric acid 1cH
- Isotherapic (*R. raciborskii* extract) 200cH
- Succussed water
- Water
- Baseline (from cultures neither challenged nor treated)

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

451 • *Nitric acidum* 200cH
452 • *Mercurius solubilis* 30cH
453 • Baseline (from cultures neither challenged nor treated)
454 • Succussed water

455

456 **2.5.2. Sample analyses**

457

458 The dyes used were Coumarin 7, Nile red, N,N-dimethylindoaniline (NN-DMIA), 4-
459 (bis-(4-(dimethylamine) phenyl methylene)-1(4H)-naphthalenone (BDN), Rhodanine,
460 and Methylene violet (Supplementary Material 4), all diluted in absolute ethanol (Synth,
461 Diadema, Brazil) according to the previously standardized ideal concentration [22, 32,
462 33], 24 hours before carrying out the tests for stabilization. Before use, all dyes were
463 filtered through a 0.22-micrometer mesh filter (Merck–Millipore, Darmstadt, Germany).

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

468 Before adding into the dyes, potencies were again manually succussed using 40
469 vertical movements and filtered through a 0.22-micrometer mesh filter (Merck–
470 Millipore, Darmstadt, Germany). All procedures were carried out in a laminar flow
471 cabin, whose environmental conditions were registered daily (temperature: 25.8–
472 25.9ºC; humidity: 39-43%; magnetic flow: 0.03-0.07µT). Measurements were made

with a thermo-hygrometer (Tomate PD-003, São Paulo, Brazil) and a Gaussmeter (frequency range: 30 to 300Hz, resolution 0.01 to 0.1µT, 3% precision at 50-60Hz - Instrutherm DRE 050, São Paulo, Brazil).

The spectrophotometer used in the experiment (FEMTO 800 XI, São Paulo, Brazil), with a sensitivity of 1nm, was calibrated with pure ethanol (Synth, Diadema, Brazil) to determine the baseline and used to scan the entire visible spectrum (350 to 800nm) of each testing dye to identify the specific absorbance peak for each experimental series.

The analyzes of the samples were performed in a microplate spectrophotometer (EPOCH – Agilent BioTek, Santa Clara, CA, USA), whose wavelengths were selected according to the data obtained in the visible spectrum scanning for each dye. Data were automatically generated in an Excel electronic sheet.

The homeopathic potency samples were distributed in conventional flat-bottomed 96-well microplates for ELISA tests to screen responsive dyes in the first round. Each sample was distributed in eight wells per plate, four plates per dye. In each plate, a row of eight wells containing only absolute ethanol and another containing only pure dye (diluted in alcohol, as indicated in Supplementary Material 4) was prepared. In the following rows, samples, and dyes were inserted into each well, 236µl of dye and 4µl of sample, so the ratio between them was 1:60 [22, 32, 33]. The absorbance delta represented each sample absorbance minus the pure dye absorbance average per plate, already discounting the natural absorbance of absolute ethanol.

Considering the recent results obtained by Cartwright in 2020 [37], in which evidence for the electromagnetic nature of the interaction dye/potency was presented, the tests were carried out in two stages, the first being carried out as described above and the second carried out after submitting samples to a constant magnetic field for 15

497 minutes, using a neodymium magnet model N42 – NdFeB (Magnetum Produtos
498 Magnéticos, São Paulo, Brazil), measuring 5.08 x 5.08 x 1.27cm, in a 270g block
499 format, coated with nickel, whose capacity is 2400 Gauss, according to manufacturer´s
500 information. The exposition time was chosen based on [52].

501 All samples of the same experiment were simultaneously subjected to the
502 magnetic field. The microtubes were placed at the bottom of a Becker and positioned
503 in the same direction of the magnetic flow, as shown in Supplementary Material 5. The
504 magnet was set on a bench free of equipment plugged into the electric current to avoid
505 interference, and cell phones were kept at least 2 meters away.

506 After analyzing the interaction between potencies and dyes, the most expressive
507 results were obtained after passing the samples through the magnetic field with greater
508 significance and a lower coefficient of variation. Thus, this method was chosen for the
509 following steps concerning the analysis of culture medium samples. The dyes selected
510 for these analyzes were a) Coumarin 7, responsive to the Isotherapic (*R. raciborskii*
511 extract) 200cH, *Plumbum metallicum* 6cH, and *Nitric acidum* 6cH; b) Nile red,
512 responsive to *Nitric acidum* 200cH and *Mercurius solubilis* 30cH.

513

514 **2.6. Analyses of physicochemical parameters of *R. raciborskii* cultures**

515

516 Cultures of *R. raciborskii* in ASM-1 medium were used in this assay, as described
517 in item 2.4. Based on previously obtained results*, Nitric acidum 200cH* and *Mercurius*
518 *solubilis 30cH* were used for treatment. Sucussed water was used as vehicle control,
519 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 the culture containing 3×10^7 filaments per liter were inserted into 250ml Beckers (N=4 per treatment) and kept in cabins with stainless steel walls and a glass side lid to allow light to pass through. Using those cabins permitted the maintenance of a protected environment during the experiments. Cold white fluorescent light fixed in the roof provided the light cycle (12h light – 12h dark). Temperature (25.7 ± 0.74ºC), humidity (65.8 ± 3.42%), and constant magnetic field (0.01µT) inside the cabins were recorded daily throughout the experiment.

Two cabins were used simultaneously: one with cultures treated with potencies not subjected to the magnetic field and the other with cultures treated with potencies subjected to a magnetic field, as described in item 2.5.2. The tests were carried out over five days. The physicochemical 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 Day 1 - the cultures were divided into 100ml aliquots and distributed in 250ml Beckers, four aliquots per treatment. In this way, 16 Beckers were placed in each cabin equally apart and covered with a filter paper lid to avoid the accidental deposition of particles suspended in the air. The first measurement of physicochemical parameters was performed on this day. Day 2 - the physicochemical parameter measurements were performed twice on this day, the first time immediately before the potencies (or control) insertion into the medium and the

second time immediately afterward. The treatments were performed by inserting 100µl of each potency in the respective Becker. Only measurements of physicochemical parameters were performed on days 3, 4, and 5. Four meters 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) and dried with soft tissue paper. Every day, before starting the experiments, the sensors were sterilized under a UV light inside a plastic envelope for 15 minutes. All measurements were made in the morning.

2.7. 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 ANOVA evaluated normal variables for identifying statistical significance among treatments, and Two-way ANOVA for identifying statistical significance among treatments in the 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, followed by Dunn´s post-test. Outliers were identified following Tukey´s rule using the Prism 9.5 tools and removed if necessary. The significance level adopted was $\alpha=0.05$.

3. Results

3.1. *R. raciborskii* extract toxicity on *Artemia franciscana*

The hatching rate in 24, 48, and 72 hours after the exposition 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, being the ideal condition to proceed with the following tests.

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*. N/A = not applicable.

Challenge	**Equivalent saxitoxin per well (ug/liter)**	**24 hours hatching rate (%)**	**48 hours hatching rate (%)**	**72 hours hatching rate (%)**
Baseline	N/A	9.82	80.00	80.37
Extract X	0.6942	9.40	85.00	85.23
Extract Y	0.4246	0	0	0
Extract Z	0.2125	0	0	0
Extract 5	**0.2086**	**5.71**	**39.00%**	**39.43**
Extract 6	0.1608	0	1.00	0.85
Extract 7	0.1393	9.09	58.00	59.89
Extract 8	0.0569	8.60	73.00	78.49

1% hydrochloric acid 0.05M (5 x 10^{-4} M)	N/A	3.95	54.00	64.47

579

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

584

585 **Table 3.** Cyst hatching and nauplii lethality rates after 48 hours of challenging the
586 function of decrescent extract 5 concentrations in seawater. The known saxitoxin
587 concentration of the raw extract was 20µg/liter, according to the number of filaments
588 produced by the cyanobacteria *in vitro*. N/A = not applicable.

589

Challenge	**Saxitoxin concentration (**µg/liter)	**Hatching rate (%)**	**Lethality rate (%)**
50% extract 5	10.0	1.6	96.67
25% extract 5	5.0	25.68	51.28
12.5% extract 5	**2.5**	**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

590

591 The chosen working dilution of extract 5 was 12.5%, whose saxitoxin concentration
592 is 2.50 µg/liter. This concentration seemed quite strategic since the World Health
593 Organization and the Brazilian Health Ministry (*MS* 2914/2011) adopted the 3.0 µg/liter
594 limit as the maximum acceptable level in public reservoirs [53]. Thus, this working
595 concentration was considered an ideal experimental condition since it was strong
596 enough to produce measurable changes in *Artemia franciscana* survival and, at the
597 same time, is related to real-world conditions. The environmental conditions registered
598 in this step were: temperature: 22.6 ± 1.94^{o}C; humidity: 60.25 ± 5.06%; magnetic flux:
599 0.01 µT (invariable).

600

601 **3.2. Screening of homeopathic potencies**

602

603 The hatching reduction and increased nauplii vitality indicate Artemia species-
604 specific bioresilience skills. In this trial, the best performance was seen after the
605 following treatments in a crescent order of effectiveness:

606 - *Plumbum metallicum* 6cH – low hatching rate, highest viability (100%), but low
607 vitality.
608 - *Nitric acidum* 6cH – low hatching rate, unchallenged comparable viability, but
609 low vitality.
610 - Isotherapic 200cH – unchallenged-like hatching and vitality rates, with the
611 highest viability (100%). This was the best performance in the screening test.

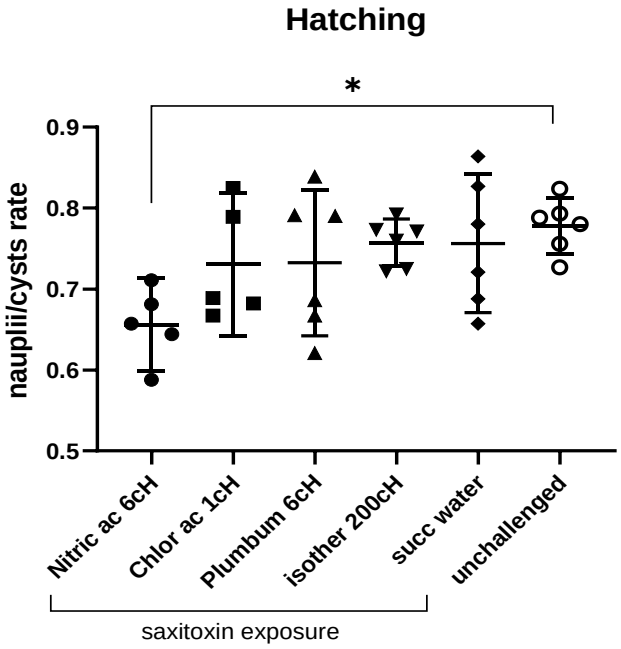
612 Detailed results from the screening of potencies and the selection of those that
613 showed evident results on cyst hatching arrest and higher vitality of the born nauplii
614 are described in Supplementary material 6.

Thus, the next step was a complete experiment with statistical analysis based on these three potencies. The methods are described in item 2.2.

3.3. Protection of *Artemia franciscana* from *R. raciborskii* extract after treatment with homeopathic potencies

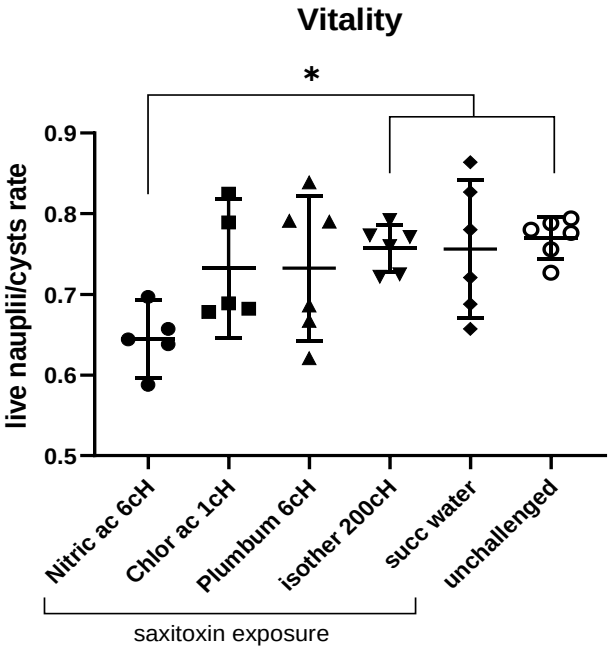
Cysts treated with *Nitric acidum* 6cH significantly decreased the hatching rate of the unchallenged samples (Figure 1) but also reduced the live nauplii/cysts ratio (vitality) (Figure 2). However, the Isotherapic (*R. raciborskii* extract) 200cH treatment led to a similar nauplii behavior about the unchallenged cysts (baseline), reproducing the same conclusion obtained in the screening phase. This effect is the most interesting since it preserves the natural behavior of nauplii besides the exposition to cyanotoxins.

Succussed water and other treatments presented higher variance in baseline and Isotherapic (*R. raciborskii* extract) 200cH treatments with no statistical significance (Figure 2).



632

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



ANOVA, F(2, 28) = 2.948, p=0.029, Tukey, * p≤0.05

638

639 **Figure 2.** Living nauplii/cysts rate (vitality) after 48 hours of exposure to extract 5
640 (saxitoxin concentration = 2.5 µg/liter) and treated with different homeopathic
641 potencies. Unchallenged nauplii correspond to the baseline. One-way ANOVA ($F_{(2, 28)}$
642 = 2.948, p = 0.029; Tukey *p≤0.05). Values represent the mean and 95% of the
643 confidence interval. Two outliers were identified.

644

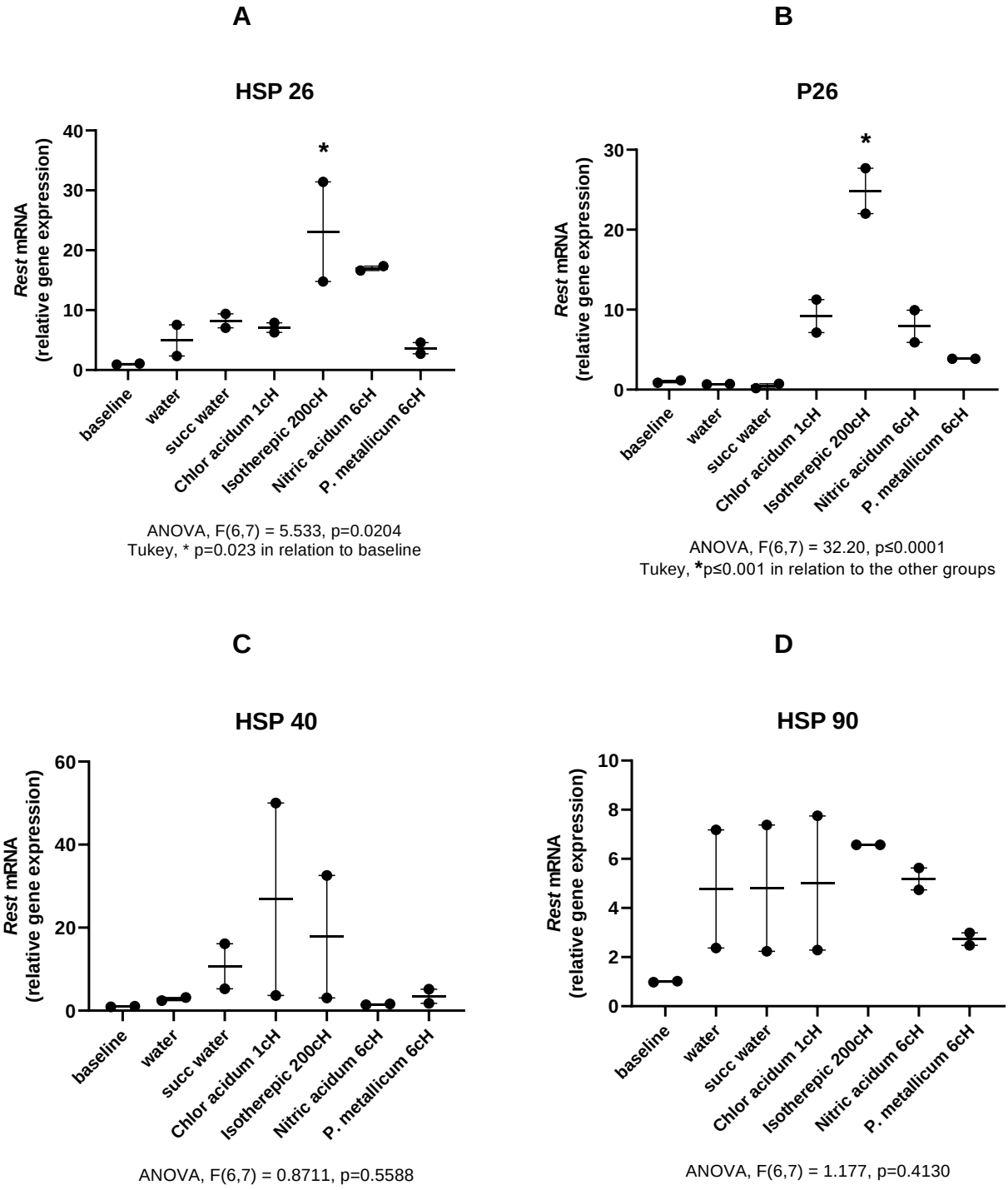
645 **3.4. HSPs gene expression**

646

647 The expression of the following genes was analyzed from the thawed biological
648 material: Hsp26, p26, Hs40, and Hsp90. There was no statistical difference in Hsp40
649 and Hsp90 expression. However, there was an increase of Hsp26 and p26 expression
650 in the groups treated with Isotherapic (*R. raciborskii* extract) 200cH (Figure 3),

651 indicating bioresilience improvement, given the role of such proteins on embryo
652 survival [12,13,54,55].

653



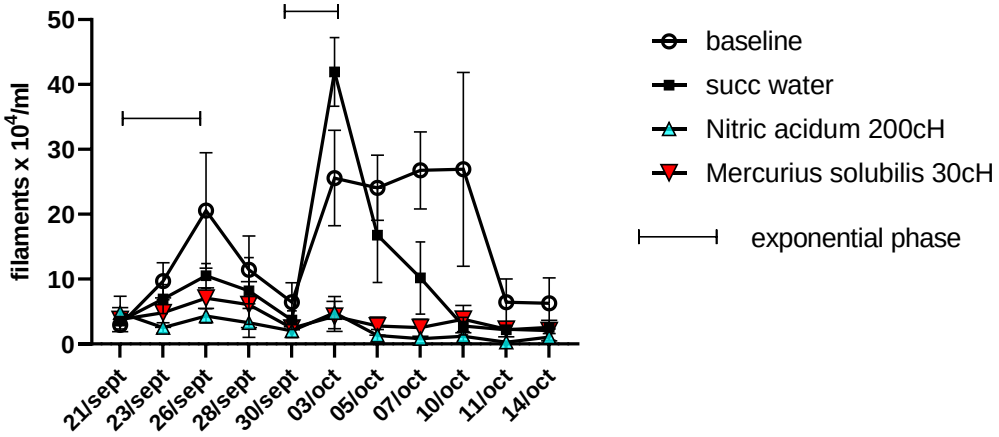
654

Figure 3. Hsp 26, p 26, Hsp 40, and expression in embryo and nauplii exposed to extract 5 of *R raciborskii* (saxitoxin concentration = 2.5 µg/liter) and treated with different homeopathic potencies. Unchallenged nauplii correspond to the baseline. Statistical data are described at the bottom of each graphic. One-way ANOVA, followed by post-test of Tukey. Values represent the mean and standard error. No outliers were identified.

3.5. *R. raciborskii* growth after treatment with homeopathic potencies

Prior to the main study, a drug screening was performed to determine which drugs were most promising in limiting the growth of cyanobacteria. *Nitric acidum* 200cH and *Mercurius solubilis* 30cH were selected for a new confirmatory assay, and succussed water was used as vehicle control (Figure 4).

Detailed results from the screening of potencies and the selection of those that showed evident results on *R. raciborskii* growth control are described in Supplementary material 6.



Mean / SEM - Two-way ANOVA (N=3)
F(30,88) = 2.791, p=0.0001 for interaction
F(10,88) = 6.001, p<0.0001 for date
F(3,88) = 23.86, p<0.0001 for treatment
Tukey, p≤0.003, Nitric acidum 200cH and Merc. solubilis 30cH *vs*. baseline and succ water
Treatments: Sept 19, Sept 26, Oct 03, Oct 10

672

673 **Figure 4.** Growth of *R raciborskii* cultures (first round) in the function of the time
674 comparing the chosen treatments (*Nitric acidum* 200cH and *Mercurius solubilis* 30cH)
675 with succussed water and unchallenged/untreated cultures (baseline). Cultures started
676 from a population of 5 x 10^7 filaments/liter, able to produce 3.6µg/ml of saxitoxin.
677 Statistical data are described at the bottom of the graphic Two-way ANOVA followed
678 by a post-test of Tukey. Values represent mean and standard error. Samples were
679 done in triplicate, and no outliers were identified.

680

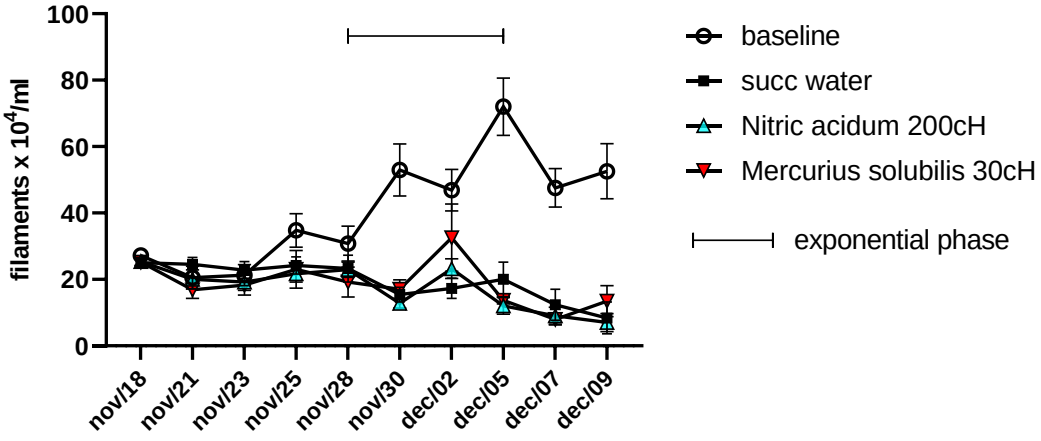
681 The baseline group of cyanobacteria cultures represents the unique condition
682 where no treatment or interventions were used, and bacteria have grown
683 spontaneously. An apparent biphasic curve is seen, which is expected for this species
684 when cultivated in ASM-1 medium. Cyanobacteria grow in time-dependent serial
685 cycles, faster or slower, depending on the environmental conditions [7].

On the other hand, cultures treated with *Nitric acidum* 200cH and *Mercurius solubilis* 30cH over two periods of exponential growth did not show growth peaks compared to the baseline. Treatment with succussed water maintained a partially limited growth 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, being $p<0.0001$. This means that the treatment effect changed as a function of time, given the stationary and exponential phases observed during the experimental period (Figure 4).

The second test checked the previous results in more challenging conditions, with more repetitions. The results are shown in Figure 5.

In this case, only a late peak of exponential growth was seen 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 essential factor for clear evidence of growth inhibition.



Mean / SEM - Two-way ANOVA (N=9)
F(27,320) = 6.069, p<0.0001 for interaction
F(9,320) = 3.213, p=0.001 for date
F(3,320) = 66.89, p<0.0001 for treatment
Tukey, p≤0.0001, Nitric acidum 200cH, Merc. solubilis 30cH and succ water *vs.* baseline
Treatments: Nov 14, Nov 21, Nov 28, Dec 05

705

706 **Figure 5.** Growth of *R raciborskii* cultures (second round) in the function of the time
707 comparing the chosen treatments (*Nitric acidum* 200cH and *Mercurius solubilis* 30cH)
708 with succussed water and unchallenged/untreated cultures (baseline). Cultures started
709 from a population of 20 x 10^7 filaments/liter, able to produce 14.6 µg/ml of saxitoxin.
710 Statistical data are described at the bottom of the graphic. Two-way ANOVA followed
711 by post-test of Tukey. Values represent mean and standard error. N=9 cultures per
712 treatment. One outlier was identified on Nov/25.

713

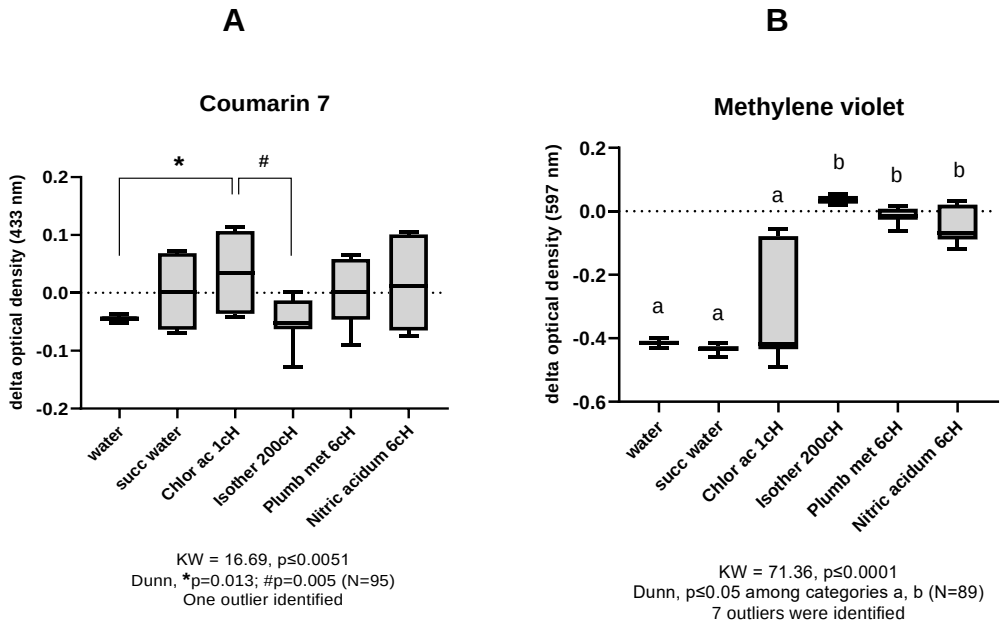
714 **3.6. Analysis of homeopathic potencies using solvatochromic dyes**

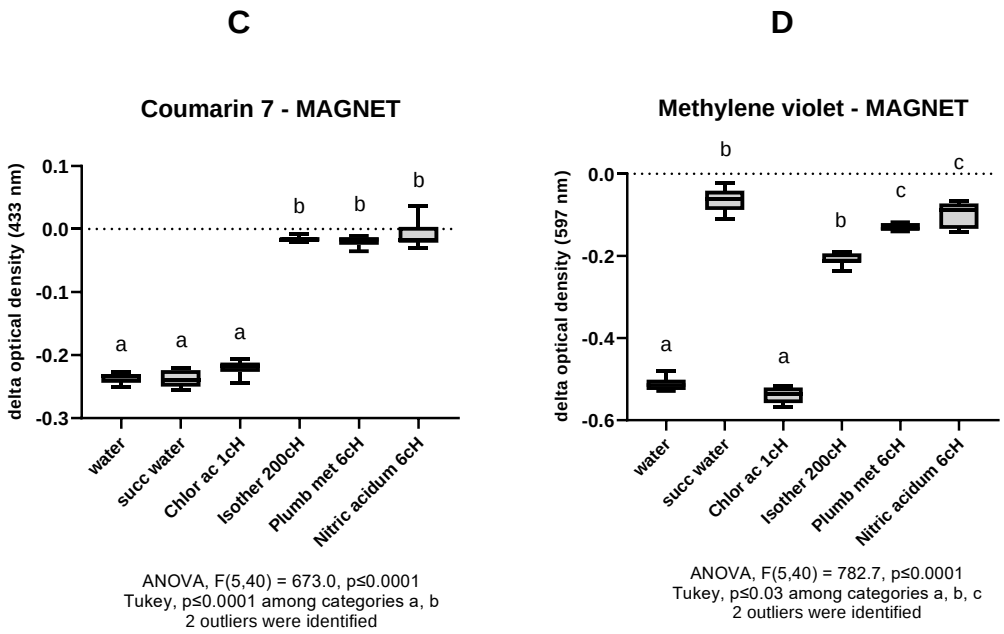
715

716 After the screening (Supplementary Material 7), *Plumbum metallicum* 6cH, *Nitric*
717 *acidum* 6cH, and Isotherapic (*R. raciborskii* extract) 200cH, used in treating *Artemia*
718 *franciscana*, showed better interaction with Coumarin 7 and Methylene violet in relation
719 to the other dyes. This effect was sharper and more specific after submitting the

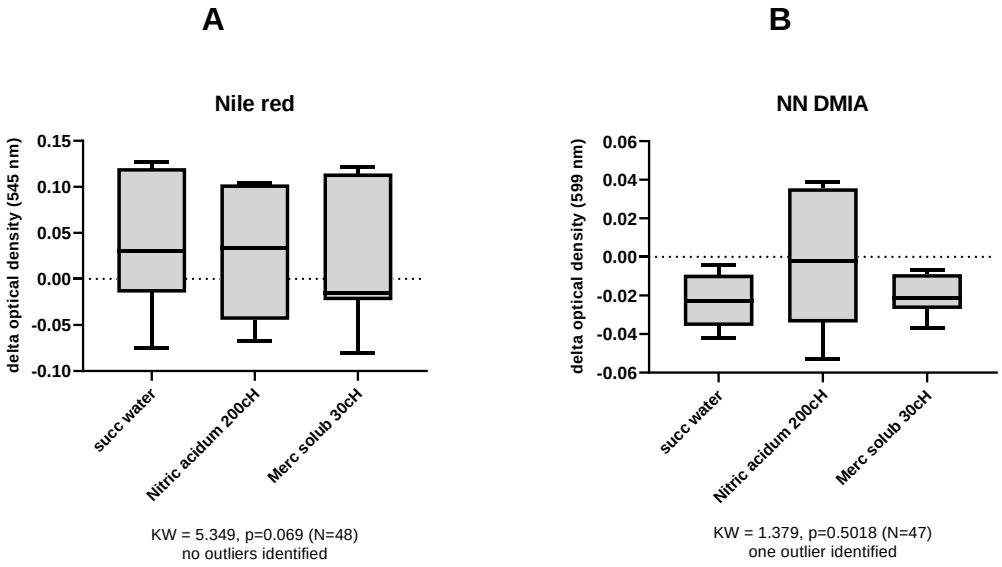
720 samples to the magnetic field (Figure 6). Considering the whole data set, the method
721 that best identified the homeopathic potencies compared to controls was the
722 interaction of homeopathic potencies with Coumarin 7 when previously subjected to
723 the magnetic field. Thus, Coumarin 7 was chosen for the evaluation of seawater
724 samples.

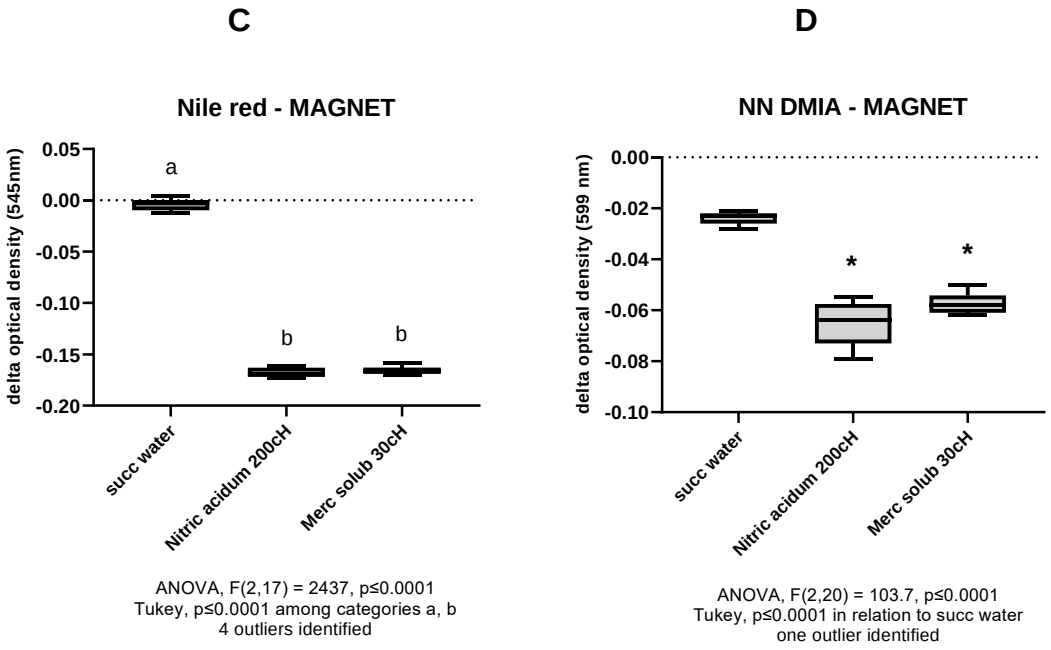
725 *Nitric acidum* 200cH and *Mercurius solubilis* 30cH, used in treating *R. raciborskii*
726 cultures, showed a better interaction with Nile red. The results were equally sharper
727 and more specific after submitting the samples to the magnetic field (Figure 7). In the
728 whole data set, Nile red showed less variance and more striking results than NN-DMIA.
729 Therefore, it could be considered a good marker for *Nitric acidum* 200cH and *Mercurius*
730 *solubilis* 30cH when previously submitted to the magnetic field. There was a clear
731 correspondence between the interaction with Nile red and the effects of these
732 potencies on the *R. raciborskii* growth control. Thus, this dye was selected for the
733 culture medium samples analyses.





734 **Figure 6.** Boxplot of Coumarin 7 and Methylene violet absorbance after interacting with
735 Isotherapic (*R. raciborskii* extract) 200cH, *Plumbum metallicum* 6cH, *Nitric acidum*
736 6cH, and controls. Kruskal-Wallis / Dunn was used in A and B since the variables
737 presented no normality at the Shapiro-Wilk test. One-way ANOVA / Tukey was used
738 in C and D since the variables presented normality at the Shapiro-Wilk test. Statistical
739 data are described at the bottom of the graphic. N=8 per treatment.





740 **Figure 7.** Boxplot of Nile red and NN-DMIA absorbance after interacting with *Nitric*
741 *acidum* 200cH, *Mercurius solubilis* 30cH, and controls. Kruskal-Wallis / Dunn was used
742 in A and B since the variables presented no normality at the Shapiro-Wilk test. One-
743 way ANOVA / Tukey was used in C and D since the variables presented normality at
744 the Shapiro-Wilk test. Statistical data are described at the bottom of the graphic. N=8
745 per treatment.

746

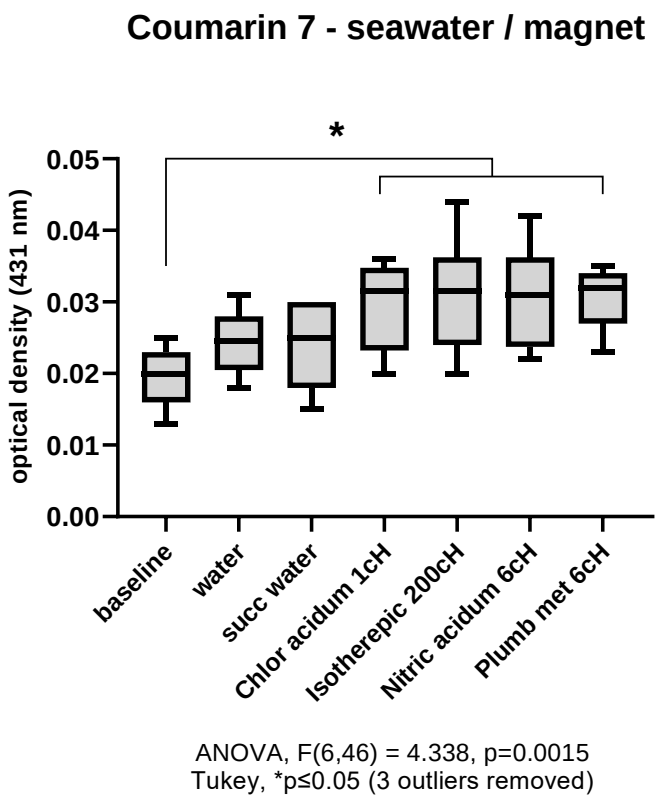
747 **3.7. Analysis of seawater and ASM-1 medium samples with solvatochromic**
748 **dyes**

749

750 After testing dyes with different potencies, Coumarin 7 was the ideal dye for
751 analyzing seawater sample pools. Each pool corresponded to a row of wells subjected
752 to the same treatment in two different saxitoxin concentrations. Samples were
753 analyzed in quadruplicate and subjected to a constant magnetic field immediately

754 before interacting with the dye. All homeopathic potencies were responsive to
755 Coumarin 7, including hydrochloric acid 1cH, as shown in Figure 8.

756



757

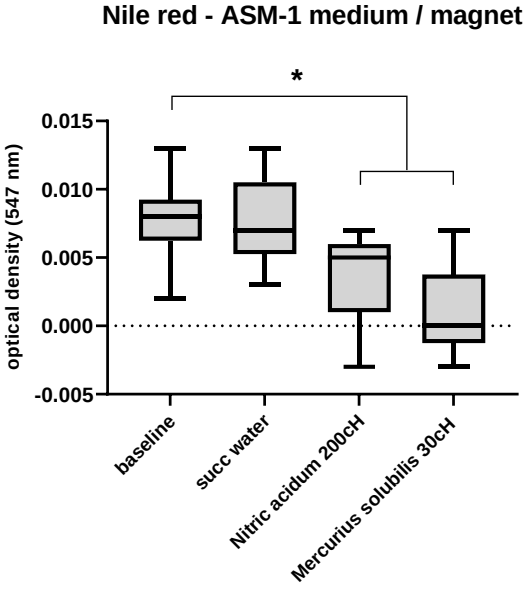
758 **Figure 8.** Boxplot of Coumarin 7 absorbance after interacting with Isotherapic (*R.*
759 *raciborskii* extract) 200cH, *Plumbum metallicum* 6cH, *Nitric acidum* 6cH, hydrochloric
760 acid 1cH, and controls. One-way ANOVA / Tukey was used since the variables
761 presented normality at the Shapiro-Wilk test. $F_{(6,46)} = 4.338$, p=0.0015, Tukey *p≤0.05.
762 Data show the combination of two pools of water, analyzed in quadruplicate.

763

764 Likewise, Nile red was the ideal dye for analyzing cyanobacterial culture medium.
765 The samples were analyzed in experimental quadruplicates for each study stage
766 (screening and repetition). Moreover, testing triplicates or quadruplicates were done

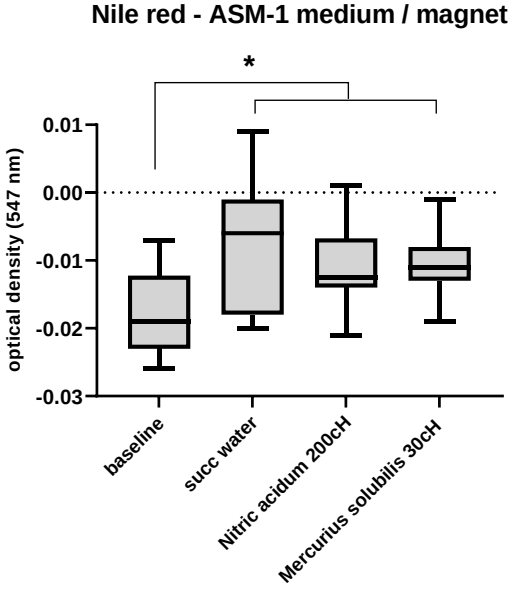
767 for the spectrophotometric reading, resulting in N=12 for the screening samples and
768 N=16 for the repetition samples. The results can be seen in Figures 9 (A and B).

A



ANOVA, F(3,39) = 11.34, p=0.0001
Tukey, *p≤0.02 (5 outliers identified)

B



ANOVA, F(3,56) = 6.835, p=0.0005
Tukey, *p≤0.03 (4 outliers identified)

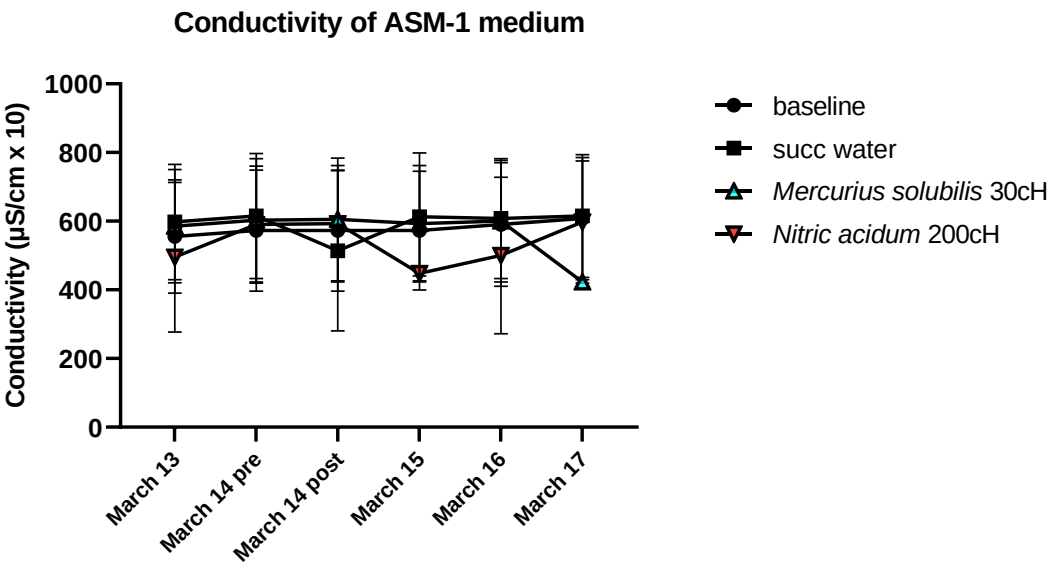
Figure 9. Boxplot of Nile red absorbance after interacting with *Nitric acidum* 200cH, *Mercurius solubilis* 30cH, and controls. One-way ANOVA / Tukey was used since the variables presented normality at the Shapiro-Wilk test. (A) Samples obtained from the screening test, $F_{(3,39)} = 11.34$, $p=0.0001$, Tukey *$p \leq 0.02$, N=12. (B) Samples obtained from the repetition test, $F_{(3,56)} = 6.835$, $p=0.0005$, Tukey *$p \leq 0.03$, N=16.

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.

3.8. Analysis of physicochemical parameters of ASM-1 medium before and after the treatment of *R. raciborskii* with homeopathic potencies

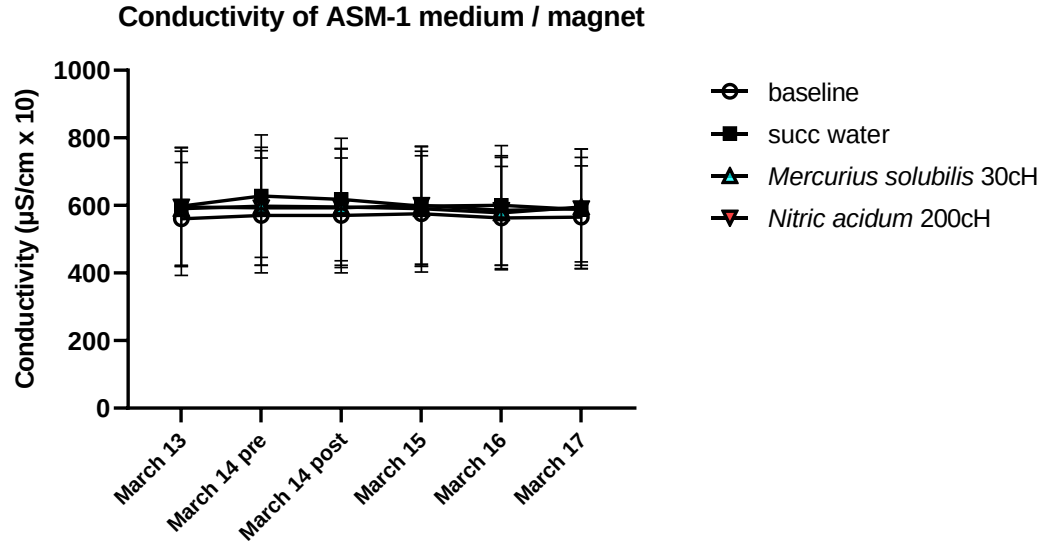
No changes in conductivity were observed as a function of treatments and time (there was no statistical interaction between them), regardless of the homeopathic potencies submission to the magnetic field, as shown in Figure 10.

A



Mean / SEM - Two-way ANOVA (N=4)
F(15,72) = 0.1119, p=0.9999 for interaction
F(5,72) = 0.02759, p=0.9996 for date
F(3,72) = 0.1116, p=0.9530 for treatment
one outlier identified in March 17

B

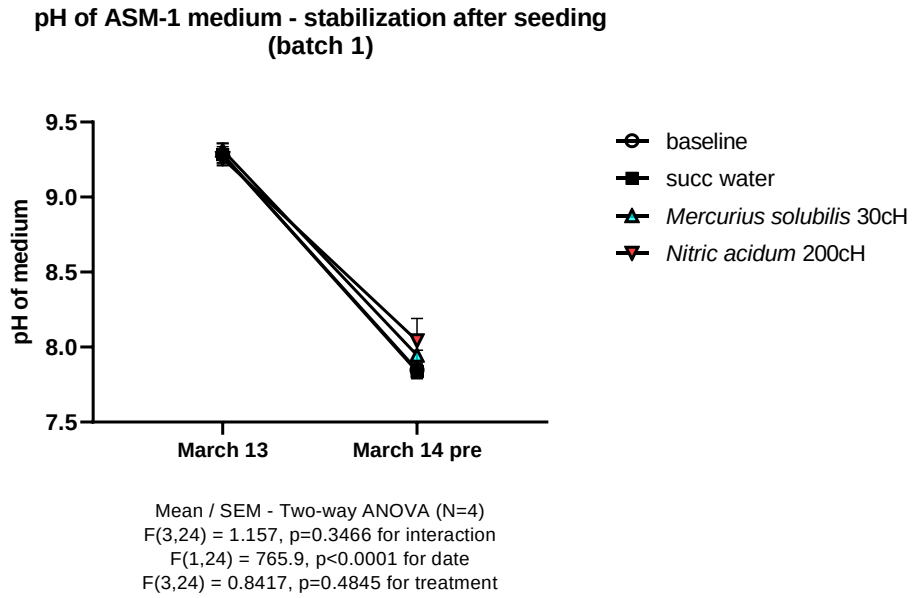


Mean / SEM - Two-way ANOVA (N=4)
F(15,72) = 0.0021, p=0.9999 for interaction
F(5,72) = 0.0049, p=0.9999 for date
F(3,72) = 0.0509, p=0.9847 for treatment

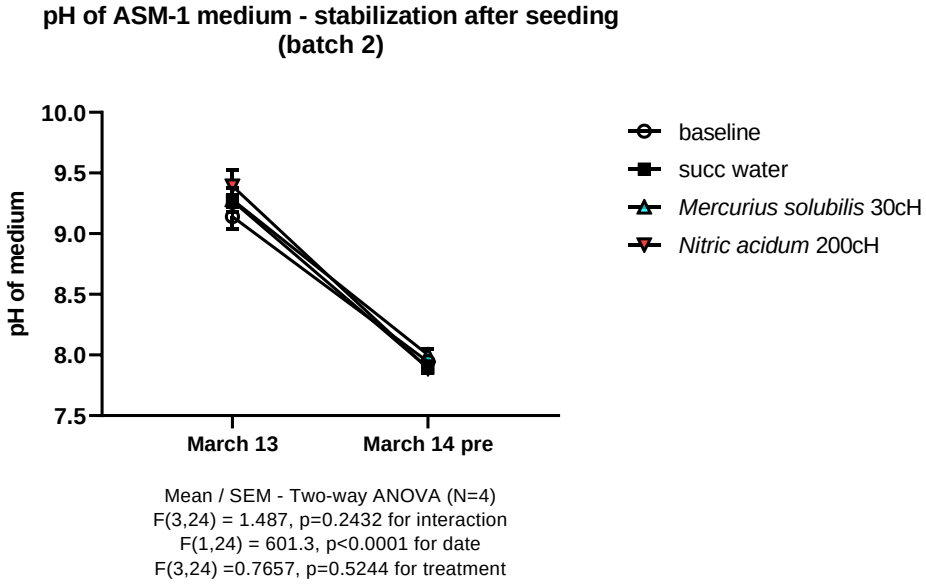
Figure 10. Conductivity variation of *R. raciborskii* culture medium submitted to different treatments (*Nitric acidum* 200cH and *Mercurius solubilis* 30cH) and controls as a function of time. (A) the time-dependent curve of cultures treated with homeopathic potencies not subjected to the magnetic field. (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 at the Shapiro-Wilk test. Statistical data are described at the bottom of the graphic. The data generated in quadruplicates are represented by the mean and standard error.

On the other hand, pH variations showed the need to stabilize cultures in the first 24 hours after distribution in the Beckers up to reach the expected pH range for the ASM-1 medium (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) (Figure 11). Then, it was possible to perform treatments and sequential observations as a function of time.

A



B



808 **Figure 11.** pH variation of *R. raciborskii* culture medium in the first 24 hours after
809 sowing. (A) batch 1 – cultures intended for treatment with homeopathic potencies not
810 subjected to a magnetic field; (B) batch 2 - cultures intended for treatment with potency
811 subjected to a magnetic field. Two way-ANOVA / Tukey was used since the variables
812 presented normality at the Shapiro-Wilk test. Statistical data are described at the

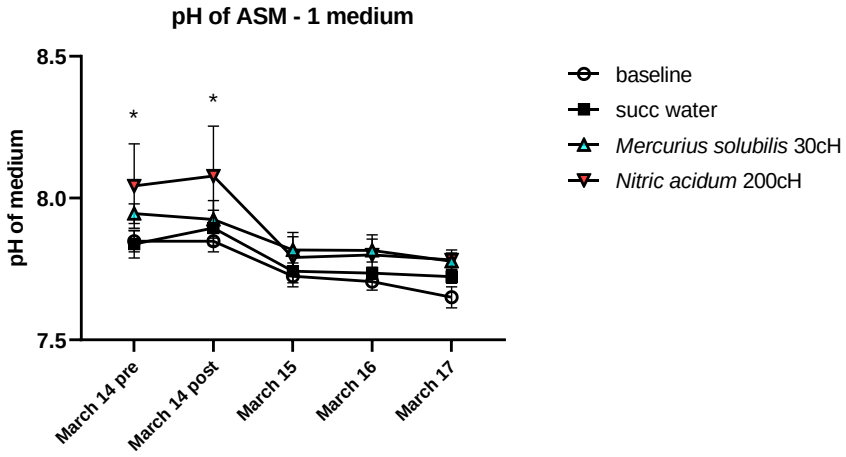
bottom of the graphic. The data generated in quadruplicates are represented by the mean and standard error.

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. However, there was a slight and transient increase in pH after the insertion of *Nitric acidum* 200cH in the culture medium, with no statistical interaction between time and treatment (Figure 12 A).

Submitting the homeopathic potencies to the magnetic field, in turn, resulted in a more precise and lasting difference between treatments and controls. In this case, both *Nitric acidum* 200cH and *Mercurius solubilis* 30cH showed the same behavior, with greater statistical significance for *Nitric acidum* 200cH. There was no statistical interaction between time and treatment, revealing a constant interference of homeopathic potencies on the medium culture independent of the time (Figure 12B).

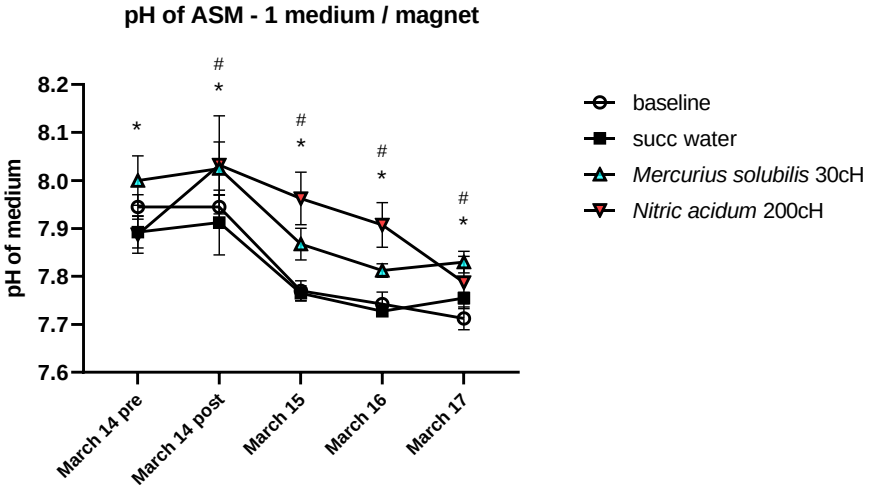
The medium temperature curve oscillated in relation to the slight variation registered in the laboratory temperature, which was adjusted to 25ºC, oscillating from 24.9 to 26.4ºC throughout the experimental period. Thus, there was statistical interaction between time and treatment, independent of submitting the samples to the magnetic field. Interestingly, there was a statistical difference between samples treated with *Nitric acidum* 200cH and those treated with *Mercurius solubilis* 30cH (up to one degree Celsius) when the homeopathic potencies were inserted in the medium. In this case, the effects were opposite depending on the magnetic field potencies submission (Figure 13 A and B).

A



Mean / SEM - Two-way ANOVA (N=4)
F(12,60) = 0.3375, p=0.9788 for interaction
F(4,60) = 7.879, p<0.0001 for date
F(3,60) = 4.597, p=0.0058 for treatment
Tukey, *p=0.0077, *Nitric acidum* 200cH *vs.* baseline
no outliers were identified

B

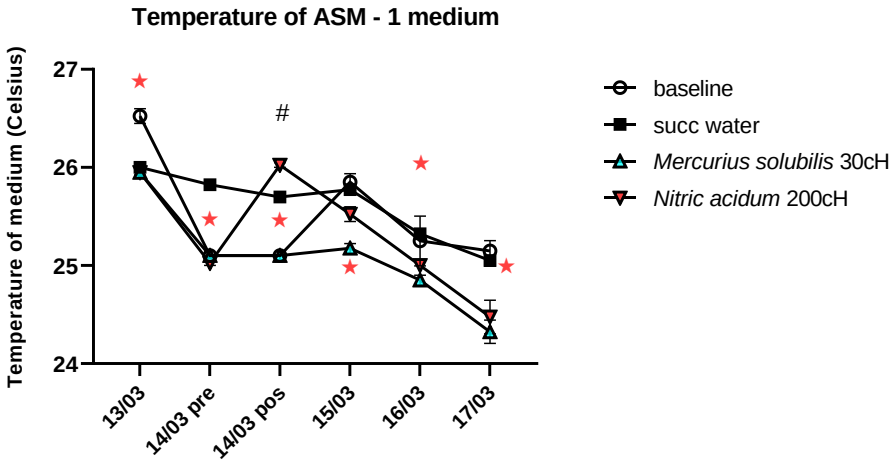


Mean / SEM - Two-way ANOVA (N=4)
F(12,60) = 1.260, p=0.2662 for interaction
F(4,60) = 17.09, p<0.0001 for date
F(3,60) = 8.247, p=0.0001 for treatment
Tukey, *****p≤0.02, *Merc. solubilis* 30cH *vs.* baseline and succ water;
#p≤0.006 *Nitric acidum* 200cH *vs.* baseline and succ water
no outliers were identified

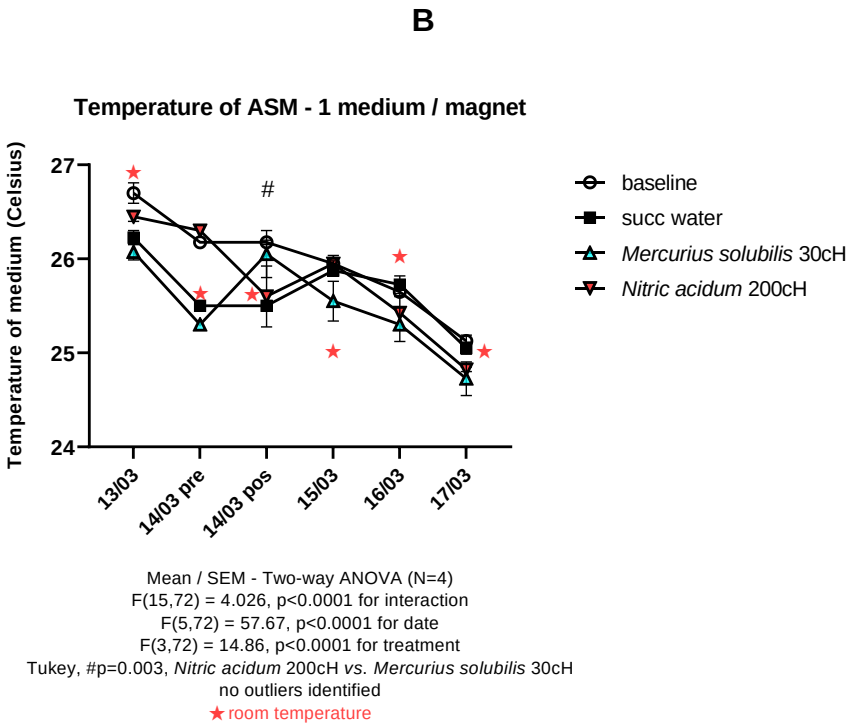
837 **Figure 12.** pH variation of *R. raciborskii* culture medium between days 2 and 5. (A)
838 batch 1 – cultures treated with homeopathic potencies not subjected to a magnetic

field; (B) batch 2 - cultures treated with potencies subjected to a magnetic field. Two way-ANOVA / Tukey was used since the variables presented normality at the Shapiro-Wilk test. Statistical data are described at the bottom of the graphic. The data generated in quadruplicates are represented by the mean and standard error.

A



Mean / SEM - Two-way ANOVA (N=4)
F(15,72) = 13.60, p<0.0001 for interaction
F(5,72) = 135.8, p<0.0001 for date
F(3,72) = 50.31, p<0.0001 for treatment
Tukey, # p≤0.0001, *Nitric acidum* 200cH *vs*. *Mercurius solubilis* 30cH
no outliers identified
★ room temperature



844 **Figure 13.** Temperature variation of *R. raciborskii* culture medium during the
845 experimental period. (A) batch 1 – cultures treated with homeopathic potencies not
846 subjected to a magnetic field; (B) batch 2 - cultures treated with potencies subjected
847 to a magnetic field. Two way-ANOVA / Tukey was used since the variables presented
848 normality at the Shapiro-Wilk test. Statistical data are described at the bottom of the
849 graphic. The data generated in quadruplicates are represented by the mean and
850 standard error. The red stars represent the simultaneous ambient temperature.

851

852 **4. Discussion**

853

854 Cyanobacteria blooms in aquatic environments have become a real and worrying
855 problem worldwide, affecting water quality and causing potential toxicity, with about 40
856 genera capable of generating cyanotoxins and producing accidental poisoning in

857 animals and humans, chronically or acutely [56,57]. There is evidence that this is a
858 growing problem, given the increasing incidence of water pollution in natural reservoirs
859 [57]. This panorama inspires and justifies the present research.

860 Eutrophication, a phenomenon related to the enrichment of nutrients in water
861 bodies by natural forces, refers to the increase in the amount of phosphorus, nitrogen,
862 and turbidity in water and the pollution caused by organic waste. One of the
863 consequences of eutrophication is the occurrence of exacerbated cyanobacterial
864 blooms in various parts of the world [58-61].

865 It is known that homeopathy produces regulatory effects on cellular functions in
866 living systems, from microorganisms to humans [22, 26, 27, 29, 62]. In view of this
867 finding, the plausibility of using homeopathy to facilitate bio-resilience of ecosystems
868 and promote global health (the FAO's "one health" approach) is proposed. Using this
869 approach, human health is not addressed without addressing animal and
870 environmental health at the same time [63]. Given its current relevance, the "single
871 health" aim has been considered a priority by the FAO [64]. Consequently, studies on
872 "homeopathy in environmental health" have been the focus of interest for our group in
873 recent years using brine shrimp as a model, given its natural capacity for bioresilience
874 [22-24].

875 In the present study, we used three interrelated experimental approaches to verify
876 the plausibility of homeopathy to improve the condition of water, containing
877 cyanobacterial colonies. The first was focused on the question: "Could homeopathic
878 potencies improve the resilience of brine shrimp exposed to cyanotoxins"? The second
879 aimed to answer the question: "Could homeopathic potencies regulate the growth of
880 cyanobacteria even in a favorable environment for their growth"? And the third question

was specifically aimed at least partially elucidating 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, 65-67]. Extract number 5 was previously obtained by dilution of a very concentrated *R. raciborskii* T3 strain extract named “extract 5”, which had a known identified saxitoxin concentration. After a series of pilot tests with *Artemia franciscana* was chosen to define a standard saxitoxin concentration close to the safety limits defined by the Brazilian Health Ministry [53] to be used in the following stages of this study.

As for the enhancement of *Artemia franciscana* embryo bioresilience by homeopathic potencies, three samples showed interesting preliminary results in terms of 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 the Isotherapic (*R. raciborskii* extract) 200cH prepared from extract 5 of *R. raciborskii*, both in the screening phase and the main experiment. In this case, the cysts hatched at the same rate as the non-exposed “baseline” group. Moreover, the vitality of the born nauplii was also comparable to the "baseline.” Vitality was defined as the number of live nauplii capable of swimming continuously concerning the number of cysts. This result denotes an extraordinary level of bioresilience, in which the natural behavior of the nauplii was preserved even after the exposition to cyanotoxins. Therefore, it was necessary to investigate the mechanisms involved through Hsp (Heat Shock Proteins) gene expression, which

actively participates in bioresilience processes in the genus *Artemia* [11-15, 54, 55, 68-69].

An increase in Hsp 26 and p26 gene expression was seen in cysts and nauplii treated with Isotherapic (*R. raciborskii* extract) 200cH compared to the other treatments. The protein p26 is a small heat shock protein abundant in *Artemia franciscana* embryos during diapause. It is responsible for prolonging it, increasing the tolerance of embryos to stress even without modifying their metabolic activity [54, 55].

Heat Shock Proteins (Hsp) 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 [70, 71]. 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 [55, 68]. 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 saxitoxin concentration. However, succussed water had a transitory effect face to the multiple growth cycles of cyanobacteria as a function of time. The ASM-1 medium is ideal for facilitating its growth, containing various metals such as iron, copper, cobalt, and molybdenum, as well as sodium and potassium. The presence of these metals in

930 water is a factor that favors cyanobacteria growth; for this reason, their presence and
931 flowering is often an indicator of pollution [56-61].

932 The quantitative evaluation of the growth rate was made from the filament count.
933 Cyanobacterial filaments are like true multicellular organisms, as nitrogen and carbon
934 are exchanged among cells through the filament's septal junctions, which ensures their
935 continuous growth. Filament counting is a commonly used technique to observe the
936 growth rate of cyanobacteria [51, 53]. In the exponential phase, for example, these
937 microorganisms are at their maximum growth capacity, and the nutrient supplies of the
938 ASM-1 medium are more than necessary for their needs.

939 It is understood that the efficiency of these homeopathic potencies under
940 laboratory conditions suggests that they may also have a fundamental role in natural
941 conditions, promoting the maintenance of water reservoirs in a eutrophic and
942 sustainable way. Such limitation of cyanobacteria growth would not prevent their
943 photosynthesis nor generate chemical residues in the water since both potencies are
944 prepared at concentrations beyond the limit of Avogadro's number. The expected
945 benefit would be improving water quality for human and animal consumption, mainly if
946 associated with other sustainable water depollution methods. Therefore, the result
947 obtained here inspires future studies in a field situation.

948 The possibility of using homeopathic preparations in field conditions leads to the
949 need for tracking their activity when inserted in large bodies of water, as previously
950 demonstrated in [35]. Thus, there is a need to identify physicochemical markers that
951 indicate their presence in the liquid medium. This seems to be possible using
952 solvatochromic dyes [34, 39]. Herein, the identification of potencies was carried out

using a wide selection of dyes that covered different ranges of the visible light spectrum to find those specific dyes that could act as ideal markers for individual potencies.

Considering the electromagnetic characteristics of the interaction between homeopathic potencies and solvatochromic dyes [36, 37], an additional protocol was proposed. The samples were subjected to a known magnetic field before being tested [40-44], thereby potentially enhancing the interaction with the dyes.

The result showed that Coumarin 7 and Nile red were the best options for identifying potencies subjected to a constant magnetic field for 15 minutes. Therefore, using the proposed protocol, these dyes were chosen to analyze *Artemia franciscana* sea water and *R. raciborskii* ASM-1 medium. The results indicated the possibility of tracking the activity of Isotherapic (*R. raciborskii* extract) 200cH in seawater using Coumarin 7, *Hydrochloric acid* 1cH, *Nitric acidum* 6cH, and *Plumbum metallicum* 6cH previously submitted to the magnetic field. On the other hand, Nile red enabled the tracking of *Nitric acidum* 200cH and *Mercurius solubilis* 30cH potencies in ASM-1 medium after submitting the samples to the magnetic field.

An important detail of the method is how samples are prepared before interacting with the dyes, as standardized in previous studies [24, 34, 35, 39]. In all cases, an additional potency (1cH) of each sample is made in a 30% filtered hydroalcoholic solution, which therefore corresponds to 99% of the content poured into the dye, respecting the 1:60 ratio, which leads to a final dilution of 1:6000, warranting that measurements were related to the solvent changes itself.

Samples of seawater and ASM-1 medium showed more significant variance than samples of pure homeopathic potencies when read in spectrophotometry even after treatment with the magnetic field, which is understandable given the heterogeneity of

977 the starting material to influence the solvent polarity after all the procedures. However,
978 this fact did not prevent the identification of potencies in the respective liquid mediums,
979 which is crucial considering the intention to use this methodology in eventual field
980 studies when large volumes of water must be managed.

981 In parallel, the additional physicochemical parameters analyzed directly in the
982 ASM-1 medium containing *R. raciborskii* (quite different medium conditions) showed
983 essential differences. Contrary to previous studies on conductivity variations in
984 different homeopathic medicines [72-76], conductivity remained constant as a function
985 of time regardless of the treatment, indicating that this is not a useful physicochemical
986 marker for homeopathic potencies in cyanobacterial cultures. The ASM-1 medium
987 composition, having high concentrations of salts and metals, probably made the
988 homeopathy introduction impossible to detect since the medium is already very
989 conductive. The repetition of this test in other experimental models, using a less
990 conductive medium, could be an ideal condition for detecting eventual changes in
991 conductivity after pouring homeopathy into the liquid.

992 On the other hand, the differences in pH among treatments seem to be clear, and
993 the samples treated with *Nitric acidum* 200cH presented a tendency to maintain a
994 higher pH in relation to the other treatments throughout the experimental period. The
995 increase in pH denotes a lower hydrogen ion concentration in the medium, which may
996 have a number of explanations, including potency-induced changes in solute and/or
997 solvent pKa values.

998 Previous studies show that pH can be a physicochemical marker capable of
999 differentiating homeopathic potencies, but in a less specific way [76]; however, even if
1000 the increase in the pH of the medium was already described in homeopathy studies

[77], this change observed after treatment with *Nitric acidum* 200cH does not seem to be associated with cyanobacteria growth inhibition since alkaline pH, instead, enhances *R. raciborskii* growth [7]. Herein, the pH oscillations did not exceed the limits between 7.0 and 9.0. Therefore, they do not compromise the water quality, which could be a relevant fact for eventual field studies. On the contrary, they favor the precipitation of heavy metals, contributing to their potability [78].

Among the physicochemical findings, the temperature oscillations when inserting *Nitric acidum* 200cH and *Mercurius solubilis* 30cH were the most peculiar. Opposite changes were observed between them, both in samples kept *in natura* or in samples previously subjected to the magnetic field. The increase in enthalpy has been reported in homeopathic preparations in a solid medium during the grinding process of metals in lactose [79, 80]. Still, a few studies have done this observation in a liquid medium [81]. The physicochemical reasons for such oscillations are still unknown, this is a topic of interest for future studies. In biological terms, it is known that *R. raciborskii* is quite tolerant to temperature variations [65-67]. Thus, the effects of homeopathic potencies on cyanobacteria growth couldn´t be attributed to a non-specific effect related to such oscillations.

The role of a constant, intense, and unidirectional magnetic flow as a facilitating factor for the interaction of the samples with solvatochromic dyes was a significant finding since it can represent an improvement of the method sensitivity. However, the impact of this interference on the biological effects of the potencies is still a matter of scrutiny, to be focused on in further studies. Moreover, variations in the magnetic field intensity and magnetic flow direction also need to be studied. Maybe it can indicate crucial factors related to the mechanism of action of homeopathic potencies in the future.

In short, the results obtained in this study are an indicative suggestion of the potential of homeopathy in mitigating environmental problems related to microorganisms that impact ecosystems, with the possibility of monitoring it by sensitive methods and further management tools. This goes toward the FAO´s recommendation for a “One Health” approach [63, 64] and the sustainable development goals for the coming decades [63].

5. Conclusion

The Isotherapic (*R. raciborskii* extract) 200cH proved to be the best option to improve the bioresilience of *Artemia franciscana* to saxitoxin, given the effects on cyst hatching, the vitality of born nauplii and Hsp26 / p26 expression. *Nitric acidum* 200cH and *Mercurius solubilis* 30cH were the best agents limiting the exponential growth of *R. raciborskii*. Concomitant increase of pH and temperature oscillations with these effects are two physicochemical parameters that deserve further studies.

In all cases, tracking the homeopathic potencies´ activity using solvatochromic dyes was possible. All physicochemical analyzes were optimized after the previous submission of the samples to a constant, intense, and unidirectional magnetic flow.

The results point to the potential of homeopathy in mitigating environmental problems related to water quality.

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Conflict of Interest: The authors declare that no conflicts of interest are related to this research.

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Supplementary material

Eco-homeopathy: Homeopathic potencies regulate the growth and toxicity of *Raphidiopsis raciborskii* (cyanobacteria) and can be tracked by physicochemically

Supplementary material 1

Quantity of *R. raciborskii* filaments per liter and the equivalent amount of saxitoxin (µg) per liter. All samples were prepared in 10 ml of 0.05 M hydrochloric acid. The correspondence was previously determined by Prof. João Sarkis Yunes at the Federal University of Rio Grande (FURG), where they were produced.

Sample (10ml HCl 0.05 M)	***R. raciborskii*** Filaments / liter (x 10^6)	(*) Equivalent saxitoxin µg/liter 0.05 M HCl
X	860	**69.42**
Y	693	**42.46**
Z	428	**21.25**
5	297	**20.86**
6	217	**16.08**
7	143	**13.93**
8	104	**5.69**
0.05 M hydrochloric acid	0	**0**

Supplementary material 2

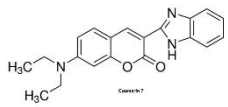
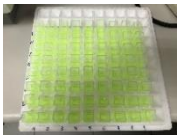
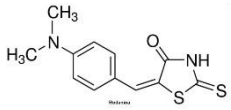
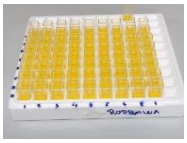
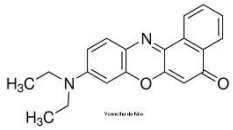
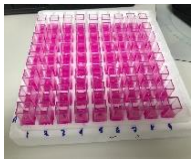
Faraday cage is built with a metallic structure covered with a 250-mesh stainless steel tissue (0.04mm wire diameter) with no opening, which protects the internal environment from any external electromagnetic oscillation (see the Gaussmeter inside). A tape containing 300 micro-led bulbs was fixed in the walls allowing constant and equally distributed light to promote cyst hatching. Lights were connected to a conventional electrical energy source outside the cage. The internal magnetic field inside the cage was 0.06 µT when the lights were on, similar to the magnetic field measured at different points of the room. The room has no windows and is well isolated from external magnetic oscillations as well. This small level of magnetic field is desirable to allow a perfect performance of homeopathic potencies on living beings, according to [48]. The cage was manufactured in the laboratory from a project designed by the physicist Prof. Adriana Ramos de Miranda.

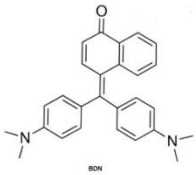
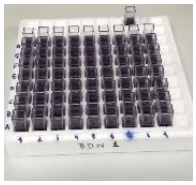
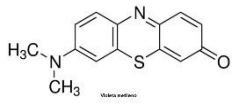
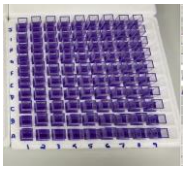
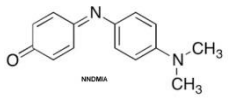
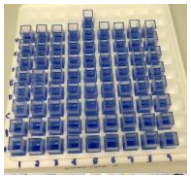
Supplementary material 3

R. raciborskii T3 strain cultures in Erlenmeyer recipient and glass tubes presenting typical yellowish color when reaching the exponentially growing phase.

Supplementary material 4

Standard solvatochromic dyes, chemical features, and respective working concentrations

Dye	Chemical structure	Color in absolute ethanol	Standard Molarity
COUMARIN 7 3-(2-BENZIMIDAZOLYL)-7-(DIETHYLAMINO)COUMARIN **CAS Number 27425-55-4** $C_{20}H_{19}N_3O_2$	 (https://www.sigmaaldrich.com/BR/pt/product/aldrich/416541)	Fluorescent green/yellow 	25 µM
RHODANINE 5-(4-DIMETHYLAMINOBENZYLIDENE) RHODANINE **CAS Number: 536-17-4** $C_{12}H_{12}N_2OS_2$	 (https://www.sigmaaldrich.com/BR/pt/product/aldrich/114588)	Yellow 	50 µM
NILE RED NILE BLUE A OXAZONE **CAS Number: 7385-67-3** $C_{20}H_{18}N_2O_2$	 (https://www.sigmaaldrich.com/BR/pt/product/sigma/19123)	Pink 	20 µM

BDN (+) 4-(BIS-(4-(DIMETHYLAMINE) PHENIL) METHYLENE) - 1(4H) - NAFTALENONE **CAS Number: not available** $C_{27}H_{26}N_2O$	 (https://www.sigmaaldrich.com/BR/pt/product/sigma/19123)	Purple/deep blue 	80 µM
VM METHYLENE VIOLET (BERNTHSEN) **CAS Number: 2516-05-4** $C_{14}H_{12}N_2OS$	 (https://www.sigmaaldrich.com/BR/pt/product/aldrich/s873705)	Purple 	50 µM
N, N-DIMETHYLINDOANILINE **(NN-DMIA)** 4-[[4-(Dimethylamine) phenyl] luminol]-2,5-cyclohexadien-1-one **CAS Number: 2150-58-5** $C_{14}H_{14}N_2O$	 (https://www.sigmaaldrich.com/BR/pt/product/sial/216313)	Blue 	25 µM

Supplementary material 5

Previous treatment of the samples with a 2400 Gauss static unidirectional magnetic field generated by a neodymium magnet to evaluate the interaction with solvatochromic dyes.

Supplementary material 6

Screening of potencies used in *Artemia franciscana* cultures:

Screening of homeopathic potencies and the respective controls for selection according to nauplii vitality (living nauplii/cyst ratio) and lethality (dead nauplii/cyst ratio), and cyst hatching ratio after the exposure to the extract 5 of *R. raciborskii* containing 2.5µg/liter of saxitoxin (N=6594 cysts). The selected potencies are **bold**.

Potencies	live / cyst ratio	dead / cyst ratio	hatching ratio
Baseline	**0.824**	**0.006**	**0.828**
Water	0.744	0.014	0.754
Succussed water	0.775	0.006	0.779
Hydrochloric acid 1cH	0.709	0.006	0.714
Isotherapic 6cH	0.717	0.037	0.743
Isotherapic 30cH	0.732	0.011	0.740
Isotherapic 200cH	**0.809**	**0.000**	**0.809**
Mercurius solubilis 6cH	0.742	0.005	0.746
Mercurius solubilis 30cH	0.675	0.010	0.682
Mercurius solubilis 200cH	0.742	0.024	0.760
***Nitric acidum* 6cH**	**0.671**	**0.006**	**0.675**
*Nitric acidu*m 30cH	0.728	0.017	0.740
Nitric acidum 200cH	0.686	0.008	0.691
Phosphoric acidum 6cH	0.707	0.006	0.711
Phosphoric acidum 30cH	0.734	0.005	0.738
Phosphoric acidum 200cH	0.737	0.016	0.748
***Plumbum met* 6cH**	**0.725**	**0.000**	**0.725**
Plumbum met 30cH	0.688	0.019	0.701
Plumbum met 200cH	0.782	0.039	0.813
Sulphur 6cH	0.751	0.030	0.773
Sulphur 30cH	0.712	0.024	0.729
Sulphur 200cH	0.767	0.037	0.795
Zincum met 6cH	0.742	0.005	0.746
Zincum met 30cH	0.714	0.035	0.739
Zincum met 200cH	0.721	0.013	0.730

OBS. Isotherapic 200cH was chosen for presenting the highest level of vitality and no death, *Nitric acidum* 6cH was chosen for presenting the lowest hatching ratio, and *Plumbum metallicum* 6cH was chosen for presenting no death.

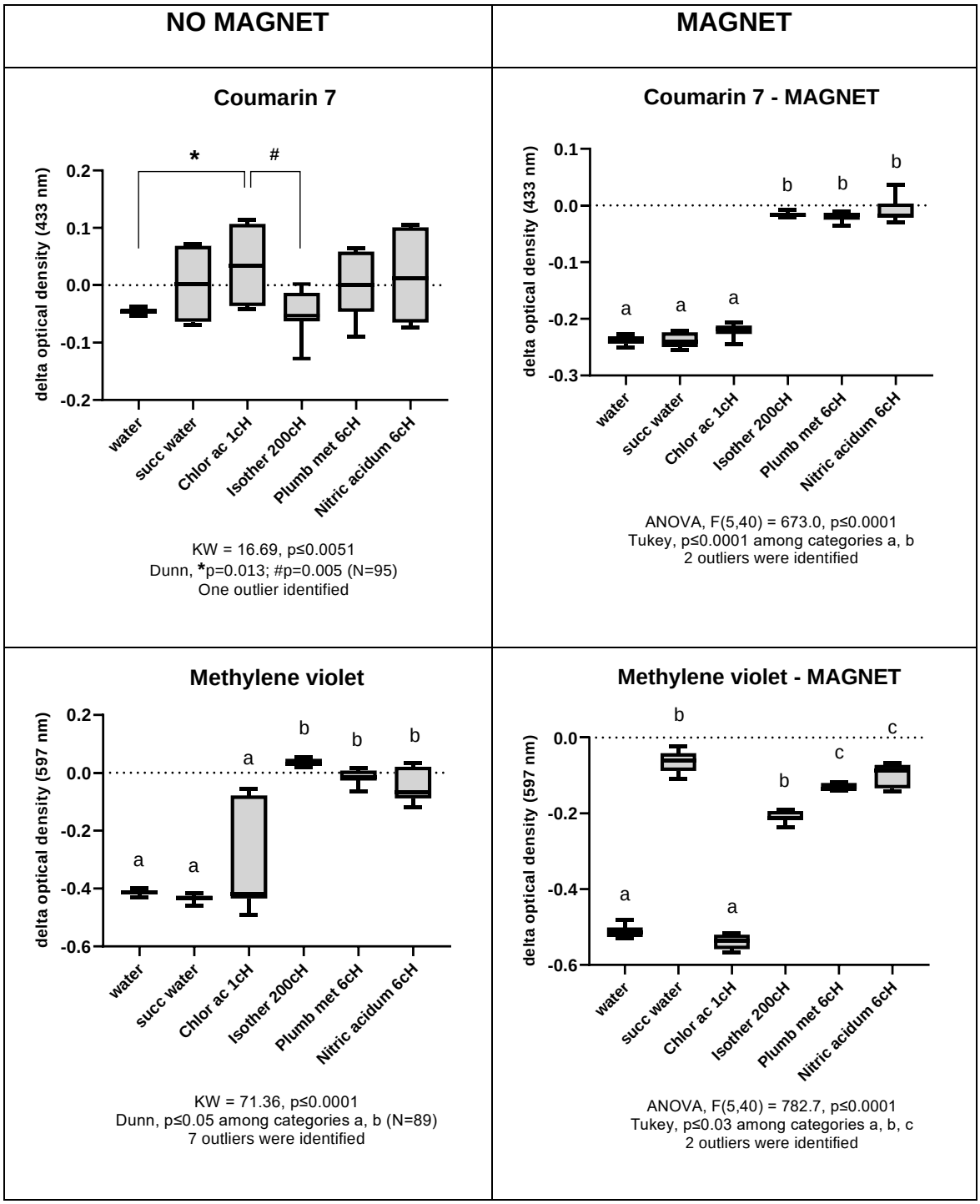
Screening of potencies used in *R. raciborskii* cultures:

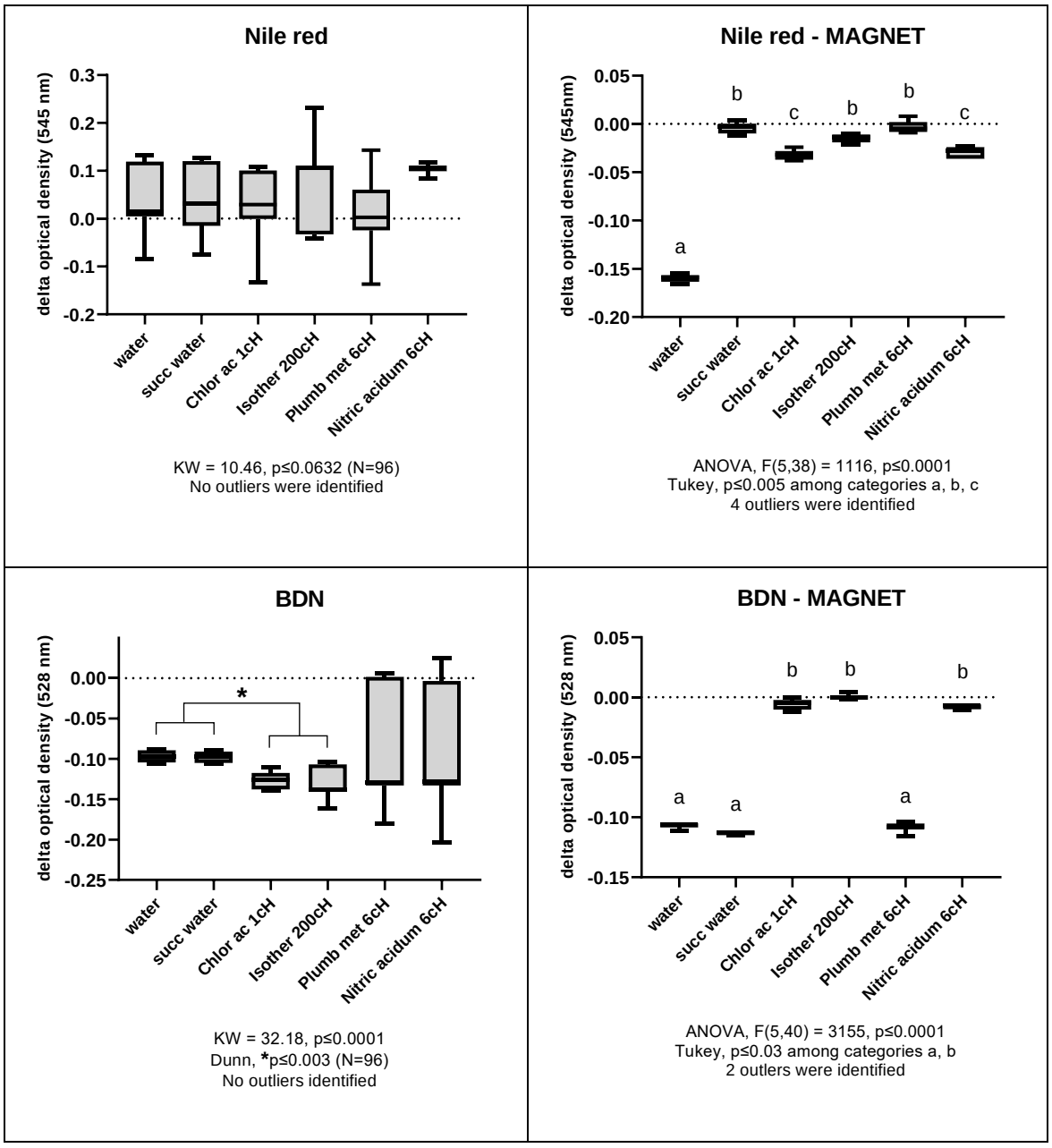
Screening of homeopathic potencies and the respective controls according to the growth limiting of *R. raciborskii* cultures. The number of filaments per milliliter was used as cyanobacteria growth parameters. Data are represented as the mean of each sample in triplicate. N=69 cultures. The selected potencies are **bold**.

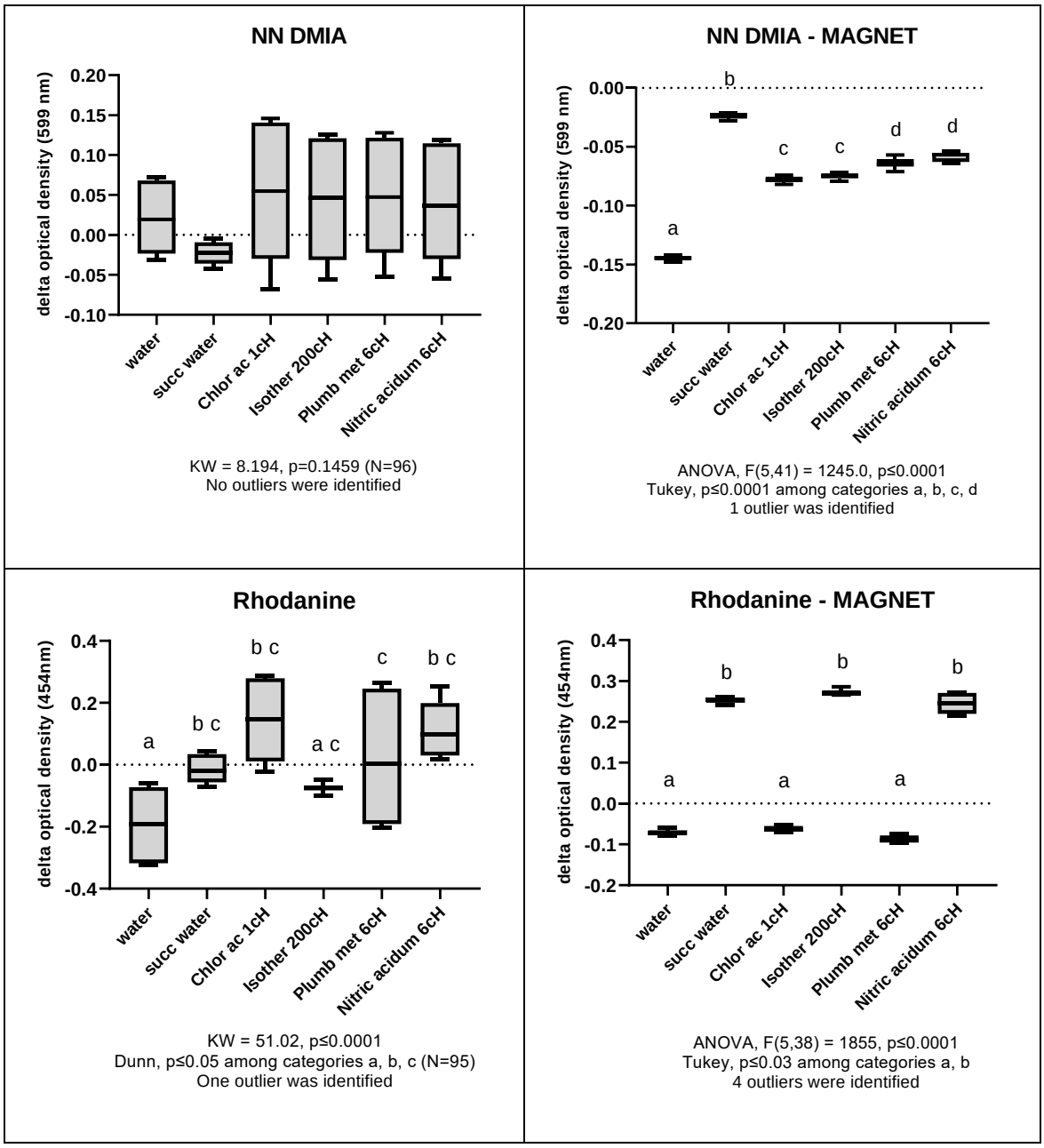
Treatments / experimental days	1	3	6	8	10	13	15	17	20	21	24
Baseline	11,67	38,67	82,33	45,67	25,67	102,33	96,33	107,00	107,67	25,67	25,00
Succussed water	14,00	27,67	42,00	32,67	14,67	167,67	67,00	40,67	11,00	8,67	10,33
Isotherapic 6cH	22,33	32,33	48,33	24,67	9,33	66,33	15,00	3,33	4,00	4,33	1,67
Isotherapic 30cH	11,67	18,33	40,67	32,00	13,33	52,67	13,67	13,00	12,00	1,33	6,00
Isotherapic 200cH	39,67	38,33	109,33	57,67	12,67	74,67	99,33	14,33	16,33	9,67	4,33
Mercurius solubilis 6cH	26,00	34,67	51,33	15,67	7,67	70,00	13,00	23,33	6,00	2,67	4,00
Mercurius solubilis 30cH	**15,00**	**19,33**	**28,33**	**24,33**	**9,67**	**17,00**	**11,00**	**10,00**	**15,33**	**9,00**	**8,33**
Mercurius solubilis 200cH	12,00	63,33	132,67	30,33	6,67	131,00	34,33	31,33	14,67	5,33	4,00
Nitric acidum 6cH	18,67	23,33	34,00	7,33	3,67	92,00	68,33	83,67	38,33	6,67	6,67
Nitric acidum 30cH	15,67	25,33	47,00	26,67	10,67	82,33	17,00	10,00	34,67	8,67	13,33
Nitric acidum 200cH	**19,33**	**10,00**	**17,33**	**13,00**	**8,00**	**19,33**	**5,33**	**3,33**	**4,67**	**1,00**	**4,33**
Phoph acid 6cH	16,33	109,67	215,00	10,67	7,67	81,33	46,33	52,67	16,33	6,67	8,00
Phosph acid 30cH	18,00	34,67	54,67	11,67	9,67	74,67	17,67	18,33	39,00	50,33	42,33
Phosph acid 200cH	17,67	40,67	98,00	13,33	12,33	43,67	25,67	21,00	40,33	42,33	36,00
Plumbum met 6cH	22,33	22,67	21,00	16,33	6,67	114,00	20,33	18,67	20,00	9,67	11,00
Plumbum met 30cH	55,67	36,33	30,67	50,00	5,33	64,00	14,33	7,67	9,00	8,67	7,33
Plumbum met 200cH	15,67	27,00	44,00	25,67	17,33	46,33	40,00	38,00	43,33	37,33	40,33
Sulpuhr 6cH	13,00	26,00	28,33	40,33	13,00	68,00	21,67	8,33	18,33	3,33	7,33
Sulpuhr 30cH	11,67	35,67	57,00	48,67	16,67	84,00	20,67	14,33	8,00	2,67	3,67
Sulphur 200cH	15,00	17,00	37,33	26,00	10,67	59,33	24,00	5,67	2,00	0,33	2,33
Zincum met 6cH	18,67	21,33	30,33	42,67	19,33	95,33	66,67	25,33	2,00	4,33	5,33
Zincum met 30cH	23,33	22,00	20,67	11,33	36,33	72,33	18,00	30,33	39,67	26,00	22,33
Zincum met 200cH	13,67	18,33	38,00	17,00	8,67	73,33	11,00	3,67	17,00	12,00	8,67

Supplementary material 7

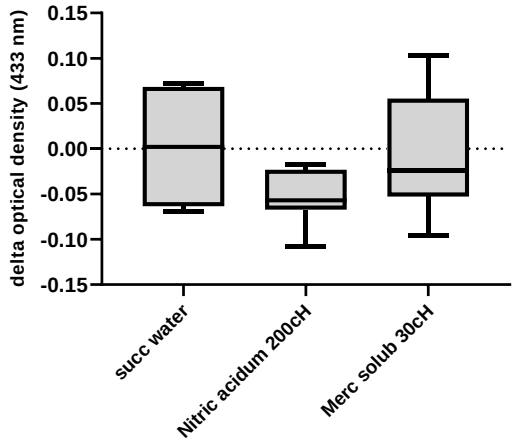
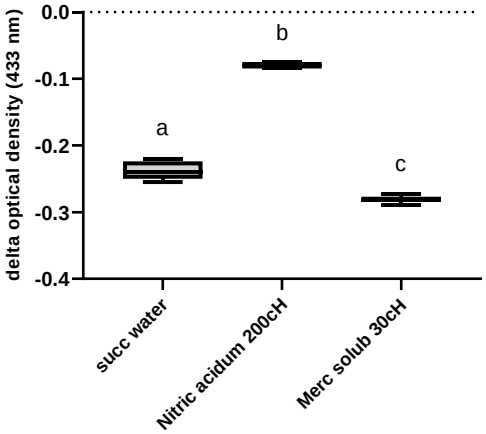
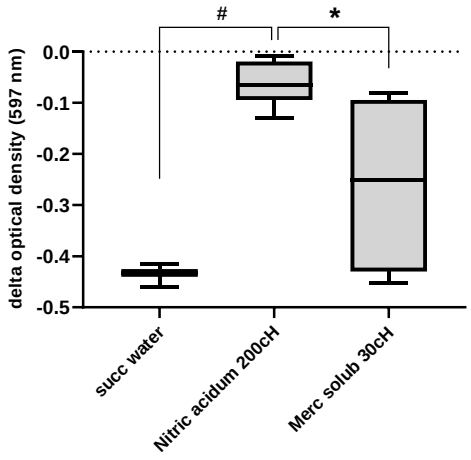
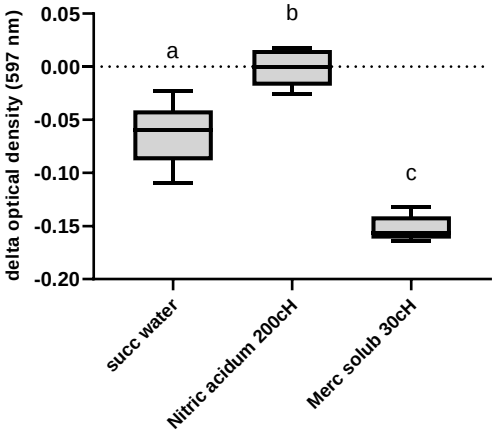
Solvatochromic dye screening for potencies used in *Artemia franciscana* cultures:

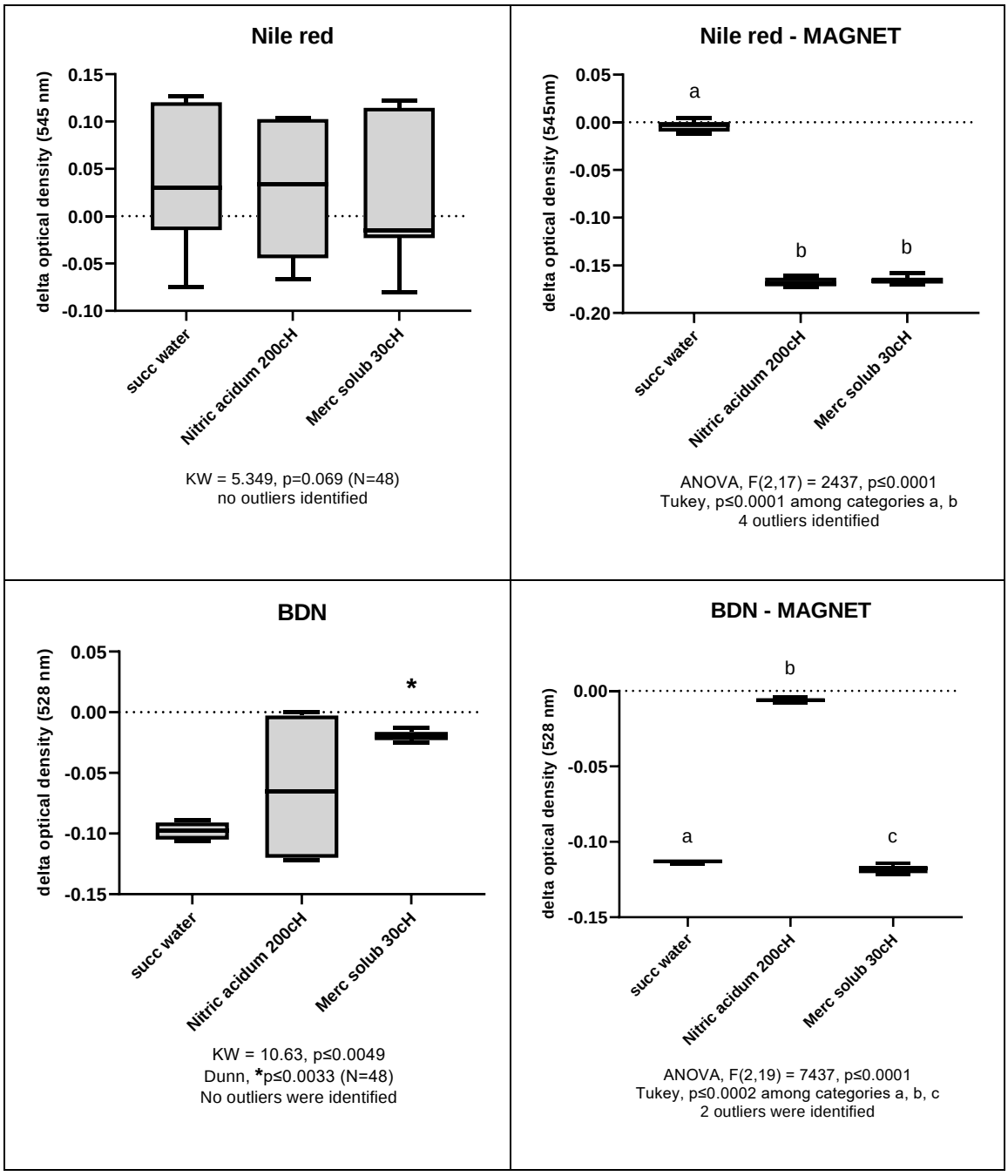


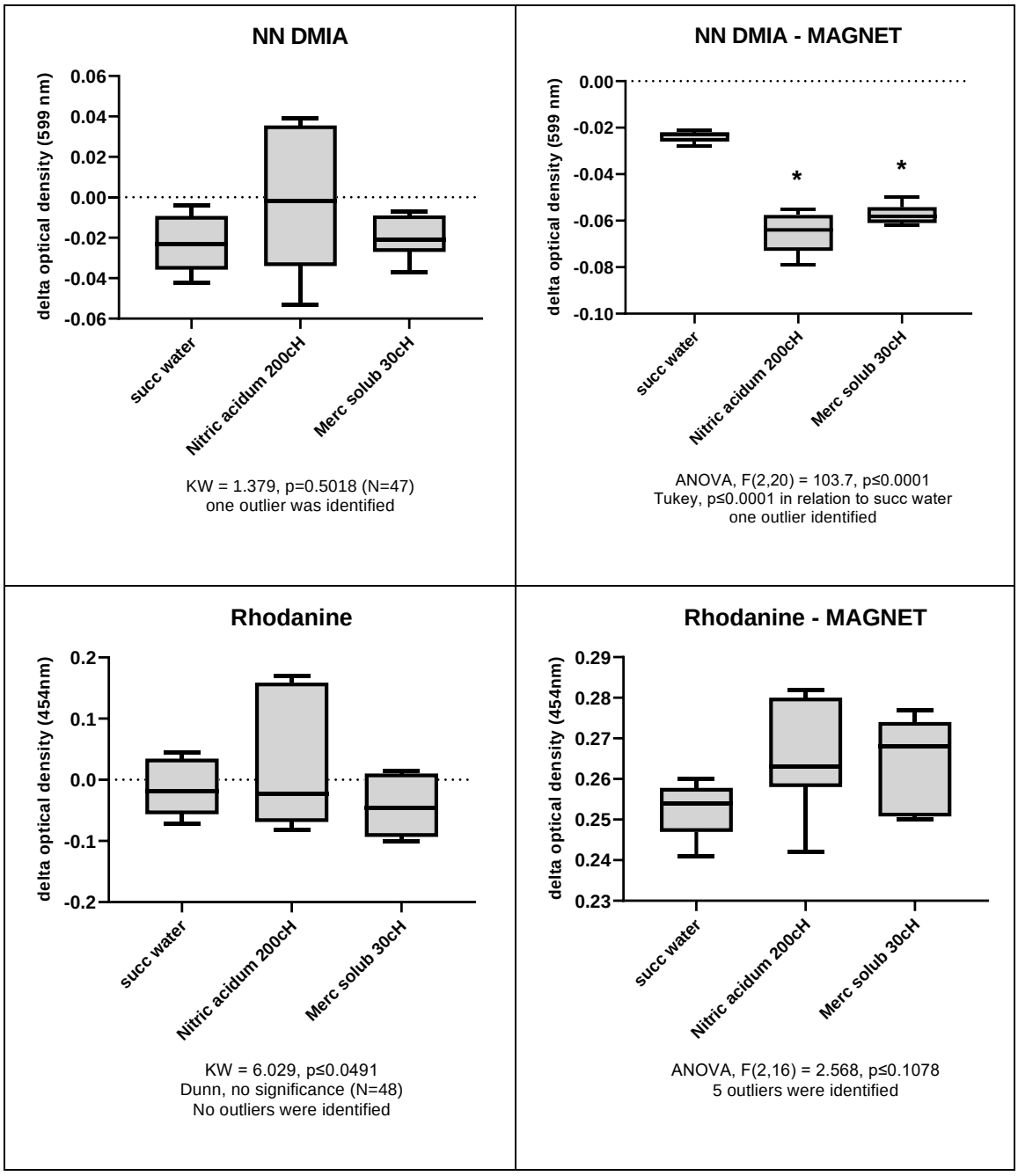

Nile red
delta optical density (545 nm)
0.3
0.2
0.1
0.0
-0.1
-0.2
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
KW = 10.46, p≤0.0632 (N=96)
No outliers were identified
Nile red - MAGNET
delta optical density (545nm)
0.05
0.00
-0.05
-0.10
-0.15
-0.20
a
b
c
b
b
c
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
ANOVA, F(5,38) = 1116, p≤0.0001
Tukey, p≤0.005 among categories a, b, c
4 outliers were identified
BDN
delta optical density (528 nm)
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
*
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
KW = 32.18, p≤0.0001
Dunn, *p≤0.003 (N=96)
No outliers identified
BDN - MAGNET
delta optical density (528 nm)
0.05
0.00
-0.05
-0.10
-0.15
a
a
b
b
a
b
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
ANOVA, F(5,40) = 3155, p≤0.0001
Tukey, p≤0.03 among categories a, b
2 outlers were identified


NN DMIA
delta optical density (599 nm)
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
KW = 8.194, p=0.1459 (N=96)
No outliers were identified
NN DMIA - MAGNET
delta optical density (599 nm)
0.00
-0.05
-0.10
-0.15
-0.20
a
b
c
c
d
d
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
ANOVA, F(5,41) = 1245.0, p≤0.0001
Tukey, p≤0.0001 among categories a, b, c, d
1 outlier was identified
Rhodanine
delta optical density (454nm)
0.4
0.2
0.0
-0.2
-0.4
a
b c
b c
a c
c
b c
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
KW = 51.02, p≤0.0001
Dunn, p≤0.05 among categories a, b, c (N=95)
One outlier was identified
Rhodanine - MAGNET
delta optical density (454nm)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
a
b
a
b
a
b
water
succ water
Chlor ac 1cH
Isother 200cH
Plumb met 6cH
Nitric acidum 6cH
ANOVA, F(5,38) = 1855, p≤0.0001
Tukey, p≤0.03 among categories a, b
4 outliers were identified

Solvatochromic dye screening for potencies used in *R. raciborskii* cultures:

NO MAGNET	MAGNET
Coumarin 7  KW = 6.240, p≤0.0442 Dunn, no significance (N=48) No outliers were identified	**Coumarin 7 - MAGNET**  ANOVA, F(2,19) = 955.5, p≤0.0001 Tukey, p≤0.0001 among categories a, b, c 2 outliers were identified
Methilene violet  KW = 29.42, p≤0.0001 Dunn, *p≤0.005; #p≤0.0001 (N=48) No outliers were identified	**Methilene violet - MAGNET**  ANOVA, F(2,17) = 73.68, p≤0.0001 Tukey, p≤0.0001 among categories a, b, c 4 outliers were identified


Nile red
delta optical density (545 nm)
0.15
0.10
0.05
0.00
-0.05
-0.10
succ water
Nitric acidum 200cH
Merc solub 30cH
KW = 5.349, p=0.069 (N=48)
no outliers identified
Nile red - MAGNET
delta optical density (545nm)
0.05
0.00
-0.05
-0.10
-0.15
-0.20
a
b
b
succ water
Nitric acidum 200cH
Merc solub 30cH
ANOVA, F(2,17) = 2437, p≤0.0001
Tukey, p≤0.0001 among categories a, b
4 outliers identified
BDN
delta optical density (528 nm)
0.05
0.00
-0.05
-0.10
-0.15
*
succ water
Nitric acidum 200cH
Merc solub 30cH
KW = 10.63, p≤0.0049
Dunn, *p≤0.0033 (N=48)
No outliers were identified
BDN - MAGNET
delta optical density (528 nm)
0.00
-0.05
-0.10
-0.15
b
a
c
succ water
Nitric acidum 200cH
Merc solub 30cH
ANOVA, F(2,19) = 7437, p≤0.0001
Tukey, p≤0.0002 among categories a, b, c
2 outliers were identified


NN DMIA
delta optical density (599 nm)
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
succ water
Nitric acidum 200cH
Merc solub 30cH
KW = 1.379, p=0.5018 (N=47)
one outlier was identified
NN DMIA - MAGNET
delta optical density (599 nm)
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
*
*
succ water
Nitric acidum 200cH
Merc solub 30cH
ANOVA, F(2,20) = 103.7, p≤0.0001
Tukey, p≤0.0001 in relation to succ water
one outlier identified
Rhodanine
delta optical density (454nm)
0.2
0.1
0.0
-0.1
-0.2
succ water
Nitric acidum 200cH
Merc solub 30cH
KW = 6.029, p≤0.0491
Dunn, no significance (N=48)
No outliers were identified
Rhodanine - MAGNET
delta optical density (454nm)
0.29
0.28
0.27
0.26
0.25
0.24
0.23
succ water
Nitric acidum 200cH
Merc solub 30cH
ANOVA, F(2,16) = 2.568, p≤0.1078
5 outliers were identified