Effects of Homeopathic Preparations of *Mercurius corrosivus* on the Growth Rate of Moderately Mercury-Stressed Duckweed *Lemna gibba* L

Tim Jäger[1,†]   Sandra Würtenberger[2]   Stephan Baumgartner[1,3]

[1] Institute of Integrative Medicine, University of Witten/Herdecke, Witten/Herdecke, Germany
[2] Scientific and Regulatory Affairs, Hevert-Arzneimittel GmbH & Co. KG, Nussbaum, Germany
[3] Society for Cancer Research, Hiscia Institute, Arlesheim, Switzerland

Address for correspondence Dr. Stephan Baumgartner, Institute of Integrative Medicine, University of Witten/Herdecke, Gerhard-Kienle-Weg 4, 58313 Herdecke, Germany
(e-mail: stephan.baumgartner@uni-wh.de).

Abstract

Background   A bioassay with severely mercury-stressed duckweed (*Lemna gibba* L.) had revealed growth-inhibiting effects of homeopathically potentised mercury(II) chloride (*Mercurius corrosivus, Merc-c.*). We hypothesised that effects of potentised preparations are dependent on the stress level of the organisms used in the bioassay. The aim of the present investigation was to examine the response of duckweed to potentised *Merc-c.* at a lower stress level.

Methods   Duckweed was moderately stressed with 2.5 mg/L mercury(II) chloride for 48 hours. Afterwards plants grew in either *Merc-c.* (seven different potency levels, 24x–30x) or water controls (unsuccussed or succussed water) for 7 days. Growth rates of the frond (leaf) area were determined using a computerised image-analysis system for day 0–3 and 3–7. Three independent experiments with potentised *Merc-c.* and three systematic negative control experiments were performed. All experiments were randomised and blinded.

Results   Unsuccussed and succussed water did not significantly differ in their effects on duckweed growth rate. The systematic negative control experiments did not yield any significant effects, thus providing evidence for the stability of the experimental system. Data from the two control groups and the seven treatment groups (*Merc-c.* 24x–30x) were each pooled to increase statistical power. Duckweed growth rates for day 3–7 were enhanced ($p < 0.05$) after application of *Merc-c.* compared with the controls. Growth rates for day 0–3 were not influenced by the homeopathic preparations.

Conclusions   Moderately mercury-stressed *Lemna gibba* L. yielded evidence of growth-enhancing specific effects of *Merc-c.* 24x–30x in the second observation period (day 3–7). This observation is complementary to previous experiments with severely mercury-stressed duckweed, in which a decrease in growth was observed in the first observation period (day 0–3). We hypothesise that the differing results are associated with the level of stress intensity (moderate vs. severe).

Keywords
► *Lemna gibba* L.
► duckweed
► stress level
► mercury
► *Mercurius corrosivus*

Introduction

Modes of traditional, complementary, and alternative medicine (TCAM) have become increasingly popular in recent decades,[1–3] also because people feel the need to live in greater balance and harmony with nature. Most TCAM methods developed centuries or even millennia ago—based on empirical observation and intuition rather than on clinical trials and scientific data.[4–7]

Due to the popularity of these methods and a general need for professionalisation, it is important to perform scientific research in this area. One major objective is retrospectively to explain why TCAM methods are rated effective and helpful by a considerable part of the general population.

Especially in the case of homeopathy there is a long-lasting debate on the scientific plausibility of its principles, in particular how the mode of action could be explained in scientific terms—mainly because there are pharmaceutical preparations with such a high degree of dilution that the probability of finding any molecules of the original ingredient is virtually zero.[8–10] Therefore, one of the most important questions in the context of the scientific evaluation of homeopathy is to determine whether there is convincing evidence for specific effects of ultra-high dilutions.

Since clinical research is very time-consuming and expensive, it is appropriate to start with basic research in less complex model systems, to determine empirical evidence for specific effects of potentised preparations, and also to establish hypotheses regarding a physico-chemical explanation of the mode of action.[11] Furthermore, less complex systems are more flexible and can be adapted to a larger number of research questions.

A major task in homeopathic basic research is to develop reliable and stable model systems delivering reproducible results. There have been high-quality studies whose results could not be reproduced subsequently; in some cases results in repeat experiments were not significant, and sometimes they were still significant but reversed.[12,13] For instance: a growth enhancement effect caused by homeopathic preparations can turn into a growth inhibition.[14–17] Various possible reasons for such failing or antagonistic effects have been discussed.[17–21] Thus there is a pronounced need for stable test systems with reproducible outcomes, and for knowledge of relevant factors influencing experimental outcome in a given test system.

In this study we worked with a test system using impaired duckweed (*Lemna gibba* L.). In ecotoxicology, duckweed is an organism often used to examine water quality. Well standardised test systems with *Lemna gibba* are used in different areas of science.[22–24] Duckweed has also been used as bioassay in homeopathic basic research.[20,21,25–27] Besides experiments with unimpaired plants,[28] models with "diseased" organisms were developed by stressing duckweed with toxic inorganic compounds.[29] A previous study in homeopathic basic research which used arsenic(V) as stressor showed stable, reproducible, and significant results.[26] To address further research questions, we have changed the stressor in the present study from arsenic(V) to mercury(II) chloride. To examine possible reasons for antagonistic results, we varied the stress level to investigate a possible influence of the artificially induced degree of stress of the organisms on experimental outcome: i.e., on the effects of homeopathic preparations. The results of three independent experiments with severely stressed duckweed (5 mg/L mercury(II) chloride over 48 hours) have already been published.[21]

In this publication we present three further independent experiments, which examine the influence of mercury(II) chloride potencies at seven potency levels (*Merc-c.* 24x–30x) on moderately impaired duckweed (poisoning by 2.5 mg/L mercury(II) chloride over 48 hours). The experimental set-up was blinded, randomised, and controlled with succussed and unsuccussed water. Furthermore, we examined the stability of the system by conducting three independent systematic negative control (SNC) experiments, comparing effects of water samples of identical origin in blinded experiments, using the randomisation code of the experiments with *Merc-c.*[30]

Materials and Methods

General Experimental Design

A single experiment comprised 60 beakers with *Lemna gibba* L. (►**Fig. 1**) that had been stressed with mercury for 48 hours. For each experimental parameter ($n = 15$ in total, $n = 14$ letter-coded samples and one open control condition, see below), four replicates were used and randomly allocated in a fixed blocked randomisation scheme. The 14 coded samples
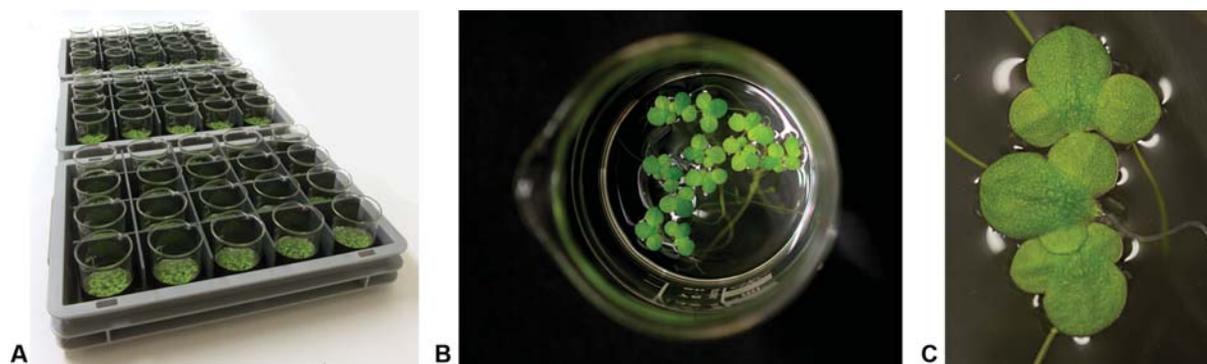

Fig. 1 (**A**) In a single experiment, 60 beakers with *Lemna gibba* were used. For each experimental parameter ($n = 15$ in total), four replicates were used and allocated in a fixed blocked randomisation scheme. The 15 experimental conditions consisted of 14 letter-coded samples and one additional open control with unstressed duckweed (the latter control was not used for statistical evaluation). (**B**) Single beaker with duckweed. (**C**) Three colonies of *Lemna gibba* L. with nine fronds (leaves).

consisted either of seven potency levels (from 24x to 30x) of *Mercurius corrosivus (Merc-c.)* or of seven independent control preparations (three samples of unsuccussed water and four samples of one-time succussed water), or—in the case of the SNC experiments—of 14 unsuccussed water samples from the same source. After preparation, all test solutions were randomised and coded (blinded) by a person not involved in the experiments. Subsequently plants grew in either potentised substances or water controls for 7 days, without any further mercury stress. Growth rate of fronds was determined for two different time intervals (day 0–3 and 3–7). Furthermore, we conducted three full-size experiments with pure water as the only treatment parameter (SNC experiments) to investigate the stability of the experimental set-up over the entire study period. Thus, six experiments were conducted in total between December 2015 and July 2018.

Preparation of Potentised Test Solutions and Controls

A detailed description of the sample preparation and cleaning procedures has been given in previous publications.[26,29] In brief, all test solutions for one experiment (potencies and controls) were freshly prepared, using the multiple-glass method, between 7 AM and 10 AM on the day of the experiment from the same batch of reverse-osmosis water (Arium 61316, Sartorius Stedim Biotech GmbH, Göttingen, Germany) prepared from tap water (Arlesheim, Switzerland).

For the potentisation process Erlenmeyer flasks of Duran glass ($\leq$6x: 250 mL, $\geq$7x: 500 mL; Schott, Mainz, Germany) were used. 15 mL of potency stock solution (0.5 g/L $HgCl_2$, Sigma-Aldrich, Buchs, Switzerland) was added to 135 mL water to produce the first potency level. Potentisation was performed according to a previously used standard proto-col[26]: the Erlenmeyer flask was first agitated once upside-down to generate a vortex; after the vortex had pacified, the flask was shaken downward a second time causing chaotic agitation of the water. These two steps were repeated 12 times for one potentisation step, with an average duration of approximately 2 minutes. For the next potency level, 15 mL of this solution was added to the next potentisation vessel containing 135 mL water and agitated in the same manner. At potency level 7x, the flask size was changed from 250 to 500 mL, and the filling volume rose to 350 mL; thus 35 mL of the former potency level was added to 315 mL of water. This process of successive 10-fold dilution and vigorous shaking proceeded until the potency step 30x was accomplished.

Two types of controls were prepared: unsuccussed water (c0) and succussed water (c1), corresponding to water 1x, shaken in the same way as the potencies described above. All controls had the same flask size and filling volume as the homeopathic samples. Three samples of unsuccussed water were prepared in three 500 mL Erlenmeyer flasks, and four independent samples of succussed water in four identical Erlenmeyer flasks. These controls were chosen according to considerations discussed in detail elsewhere.[30]

From the potencies prepared, seven potency levels (from 24x to 30x) were retained for the experiments. Together with the seven control preparations (see above), 14 samples were prepared in total. These 14 test solutions were randomised

and coded (blinded) by manual random assignment of a double-letter code from a predefined list by a person not involved in the experiments.

For the SNC experiments, all test solutions for one experiment were freshly prepared between 7 AM and 10 AM on the day of the experiment from the same batch of reverse osmosis water (Arium 61316, Sartorius Stedim Biotech GmbH, Göttingen, Germany) prepared from tap water (Arlesheim, Switzerland). Fourteen samples of unsuccussed water were prepared in fourteen 500 mL Erlenmeyer flasks.

Experimental Procedure

For the duckweed bioassay, axenic (pure) stock cultures of duckweed *Lemna gibba* L. (clone no. 9352) were grown according to a standard of the International Organization for Standardisation[22] first on solid, then in liquid-modified Steinberg medium (moStM; all ingredients Fluka, Buchs, Switzerland) to acclimatise the plants to the experimental conditions and obtain large amounts of plants under controlled laboratory conditions. The medium was changed weekly to achieve rapid, near-exponential, growth. Any restrictions on growth were avoided (e.g., through space limitations or nutrient restrictions).

After a 7-day growth period, moStM was last changed 48 hours before starting the experiment. Plants (7.5 g) were transferred to one vessel containing 2,000 mL of freshly prepared moStM with 2.5 mg/L of mercury(II) chloride ($HgCl_2$, Sigma-Aldrich, Buchs, Switzerland) added. Plants were stressed in this medium for 48 hours. Fronds that were malformed or very severely damaged were removed from the vessel 24 hours before starting the experiment.[26]

On the day of the experiment, plants without visible lesions, chlorosis, or necrosis were selected from the vessel. Test specimens were sorted according to the number of fronds, size similarity, colour, and morphology. Three plants each were used as starter culture for all beakers containing test solutions or controls.

A single experiment comprised 60 beakers (►**Fig. 1**). $N = 15$ experimental parameters were investigated in four replicate beakers each ($15 \times 4 = 60$ beakers). The 15 parameters consisted of 14 letter-coded samples (seven potency levels of a given substance and seven control preparations, see above) and one additional open control condition (one parameter) with unstressed duckweed. The latter control was not included in the statistical evaluation.

For each experiment, 50 mL of moStM was poured (Bottle-Top dispensing head, 50 mL, Brand, Wertheim, Germany) into 60 beakers each (150 mL, SIMAX, Kavalier, Sázava, Czech Republic). Then 50 mL of 14 coded samples in four replicates each was added to the 56 beakers. For the one open control condition, 50 mL reverse osmosis water (from the same batch as used for the production of the coded samples) was added to each of the four beakers.

The sorted stressed duckweed colonies were carefully put at random into 56 beakers for the coded samples. Sorted unstressed duckweed was placed into the four beakers of the open control. Frond area per beaker was measured at the beginning of the experiment (day 0), and on day 3 and 7

using a camera (D200, Nikon, Tokyo, Japan; photographic lens: AF-S Nikkor 17–55 mm 1:2.8G ED, Nikon, Tokyo, Japan) and an image processing system (medeaLAB Imaging System Count & Classify, version 6.7, Medea AV, Erlangen, Germany).

Experiments were conducted in the same plant-growth chamber used for the experiments with severely stressed duckweed,[21] specially constructed to enhance homogeneity of light intensity, temperature, and air velocity, to avoid vibrations and reduce electromagnetic fields during the experiment. Duckweed was illuminated with fluorescent lights (145 ± 4.9 µmol photons m^{-2} s^{-1} PAR, F32 T8/TL 741, Philips, Andover, United States) for 16 h/d. Mean air temperature was $20.6°C \pm 0.7°C$, mean temperature of moStM $21.5°C \pm 0.4°C$ (Endotherm, Dornach, Switzerland) and mean relative humidity was $45\% \pm 10\%$ (EBI-20-TH, Ebro, Ingolstadt, Germany).

The average growth rate per day (r) based on the measured frond area was calculated for two test intervals (day 0–3 and day 3–7) according to the equation: $r = (\ln x_{t2} - \ln x_{t1})/(t_2 - t_1)$, where x_{t1} is the observation parameter value at day t_1, x_{t2} is observation parameter value at day t_2, and $t_2 - t_1$ is the time interval between x_{t1} and x_{t2} in days. More details concerning the procedures of the duckweed bioassay have been described elsewhere.[29]

Statistical Analysis

All experiments (two series [verum and SNC] with three experiments each) yielded a total of 1008 data points (six experiments × 56 beakers × three time points) that were transformed into 672 growth-rate data values for the final statistical evaluation (day 0–3 and day 3–7). Careful experimental management ensured there were no missing data.

Data from the three SNC experiments were used to estimate the variability of the bioassay. We grouped the data of the 56 beakers of every single experiment into 14 groups of four replicates (beakers) and calculated mean values for these 14 sub-groups for frond area-related specific growth rate (day 0–3 and 3–7, each). Based on these 14 values, the coefficient of variation (CV) was calculated for the two time intervals in every single experiment.

Regarding a possible succussion effect, data of the unsuccussed (c0) and succussed water controls (c1) of experiments with potentised substances were analyzed using a two-way analysis of variance *F*-test for independent samples.

A comparison of growth rate (r) data between pooled potencies and pooled water controls (succussed and unsuccussed) was evaluated for statistical significance based on two-way analysis of variance *F*-tests for independent samples. In all statistical analyses the level of significance was $\alpha = 0.05$. An interaction term between experiment number and treatment was included in the statistical model to be able to observe possible effect-modulating factors associated with the date of the experiment. Planned comparisons were evaluated with the least significant difference (LSD) test only if the corresponding global *F*-test was significant ($p < 0.05$) (Fisher's protected LSD). This constitutes a good safeguard against type I as well as type II errors.[31]

Levene's test was conducted to determine homogeneity of variances. Data distribution was evaluated graphically by quantile-quantile plots. All data were analyzed using the software JMP Version 12 (JMP, Version 12.2.0, SAS, Cary, United States).

Results

Degree of Damage

The influence of the poisoning with mercury was determined by comparing the growth rate of the pooled data of the unsuccussed (c0) and succussed (c1) water control groups to the open control group without mercury poisoning. Averaged over all three experiments, mercury-treated duckweed exhibited an area-related growth rate (r) for day 0–3 of approximately 66.2% compared with duckweed growing without mercury ($r_{with\ mercury} \approx 0.31$ d^{-1}, $r_{without\ mercury} \approx 0.46$ d^{-1}) and for day 3–7 of approximately 87.1% ($r_{with\ mercury} \approx 0.37$ d^{-1}, $r_{without\ mercury} \approx 0.42$ d^{-1}). As expected from the reduction of mercury(II) chloride poisoning to 2.5 mg/L, the relative growth rate is higher compared with the previous study that used 5 mg/L mercury(II) chloride (50.7 and 83.9% relative growth rate for day 0–3 and 3–7, respectively).[21]

Control Experiments

The stability of the experimental set-up was investigated in three SNC experiments. These revealed very small coefficients of variation for all outcome parameters measured (1.7–4.5%, ►**Table 1**).

In the statistical analysis of the control experiments (performed in identical manner to the experiments with *Merc-c.,* see below) the global analysis of variance *F*-tests yielded no significant effects for the outcome parameter with regard to treatment (here 14 sham treatments, water only) for the two test intervals (day 0–3 and day 3–7). Thus, false-positive results caused by uncontrolled influences during the experiment (e.g., systematic errors due to spatial or temporal gradients in light intensity or temperature) could be excluded with a very high degree of certainty. Also, the analyses for interaction of treatment with experiment number for the two test intervals (day 0–3 and day 3–7) yielded no significant effects (►**Table 2**, Series SNC). This means that also the single SNC experiments did not yield false-positive results, which suggests a very stable test system.

The primary evaluation of the SNC experiments was based on the randomisation code of the *Merc-c.* experiments (allocations

Table 1 Coefficient of variation (CV) for all outcome parameters in the three systematic negative control (SNC) experiments. CV was calculated based on mean values of 14 groups of four replicates (total 56 beakers) in one experiment.

Experiment no.	CV of growth rate (r)	
	Day 0–3	Day 3–7
SNC exp. no. 1	4.53%	2.95%
SNC exp. no. 2	3.62%	1.73%
SNC exp. no. 3	3.70%	2.02%

Table 2 ANOVA analysis of the two experimental series (one with the test substance *Merc-c.* and one with systematic negative control experiments) with the independent parameters experiment number ($n = 3$ independent experiments) and treatment ($n = 2$, potencies vs. controls). Data of the seven potency levels (24x to 30x) and the seven control samples (three samples unsuccussed water, four samples succussed water) were numerically pooled into two groups each. For the analysis of the SNC experiments, the randomisation codes of the *Merc-c.* experiments was applied. Measurement parameters were frond area-related growth rates for two different time intervals (day 0–3 and 3–7). Data were normalised to the mean of the pooled water controls for every individual experiment. Significant values ($p < 0.05$, *F*-test) related to treatment effects are shown in bold.

Experimental series	Effects	*p*-Values for growth rate (*r*)	
		Day 0–3	Day 3–7
Merc-c.	1: Exp. no.	<0.001	<0.001
	2: Treatment	0.125	**0.010**
	1/2: Interaction	0.996	0.443
SNC	1: Exp. no.	<0.001	<0.001
	2: Treatment	0.214	0.224
	1/2: Interaction	0.738	0.258

Abbreviations: ANOVA, analysis of variance; SNC, systematic negative control.

of the 14×4 beakers to the seven sham treatment or seven sham control groups per experiment) in the same sequence of the experiments. To further confirm that the *Merc-c.* experiments did not generate false-positive results by chance, we additionally analyzed the data from the three SNC experiments with the randomisation from all three *Merc-c.* experiments. The results of these analyses did not yield any evidence for false-positive results due to the specific randomisation lists used for the *Merc-c.* experiments (►**Table 3**).

Succussion Effect

To analyze unspecific physico-chemical effects that occur during the succussion step of the potentisation process (increased ion dissolution from the vessel walls, pH alteration due to CO_2 dissolution, etc.), unsuccussed and succussed water controls from all experiments with potentised substances were compared, as proposed by Baumgartner et al.[30] In analysis of variance *F*-tests of growth rate data, no significant succussion effect and no significant interaction with experiment number were observed for any outcome parameter (►**Table 4**). Since succussed water (c1) did not differ from unsuccussed water (c0) in its effects on duckweed growth rate, we concluded that any unspecific effects due to the succussion procedure were negligible in this test system. Thus, as had been defined *a priori*, effects of potentised substances (see below) were compared with the pooled data from both control groups (defined as control c) to increase statistical power and to obtain an equal number of samples in the group with pooled potencies and the group of controls for statistical analysis.

Table 3 ANOVA of the three independent single SNC experiments, analyzed with the randomisation of the three *Merc-c.* experiments. Data were allocated to two groups, corresponding to the randomisation codes of the *Merc-c.* experiments (group 1: beakers corresponding to the seven potency levels [24x–30x]; group 2: beakers corresponding to the seven control samples). Measurement parameters were frond area-related growth rates for two different time intervals (day 0–3 and 3–7).

Experiment	Randomisation	*p*-Values for growth rate (*r*)	
		Day 0–3	Day 3–7
SNC Exp. no. 1	*Merc-c.* 1	0.223	0.125
	Merc-c. 2	0.295	0.352
	Merc-c. 3	0.387	0.161
SNC Exp. no. 2	*Merc-c.* 1	0.330	0.112
	Merc-c. 2	0.548	0.375
	Merc-c. 3	0.558	0.390
SNC Exp. no. 3	*Merc-c.* 1	0.351	0.303
	Merc-c. 2	0.173	0.102
	Merc-c. 3	0.788	0.248

Abbreviations: ANOVA, analysis of variance; SNC, systematic negative control.

Table 4 Comparison (ANOVA *F*-tests) of unsuccussed (c0) and succussed controls (c1) for two-time intervals (day 0–3 and 3–7). Data are from the *Merc-c.* experimental series with three independent experiments.

Experimental series	Effects	*p*-Values for growth rate (*r*)	
		Day 0–3	Day 3–7
Merc-c.	1: Exp. no.	<0.001	<0.001
	2: Treatment	0.996	0.834
	1/2: Interaction	0.720	0.705

Abbreviations: ANOVA, analysis of variance; SNC, systematic negative control.

Effects of *Merc-c.* (24x–30x, Pooled Data)

Duckweed growth-rate data (area-related growth rates for the two time intervals) for the experimental series were analyzed separately, always in full two-way analysis of variance with the independent variables treatment ($n = 2$, all potency levels vs. pooled controls) and experiment number (1–3). Results are displayed in ►**Table 2** and in ►**Fig. 2A** for the area-related growth rate (day 0–3 and day 3–7). Results of the SNC experiments are displayed in ►**Fig. 2B** for comparison.

Homeopathic potencies of *Merc-c.* enhanced the growth rate of mercury-stressed *Lemna gibba* L. compared with water controls (frond area growth rate [*r*], day 3 to 7 [$p < 0.05$], ►**Table 2**). The stress-induced growth inhibition of 12.9% for day 3–7 was decreased by 2.4% to 10.5%, averaged over all potency levels. Growth rates in the first time interval (day 0–3) were not influenced by the homeopathic treatment.
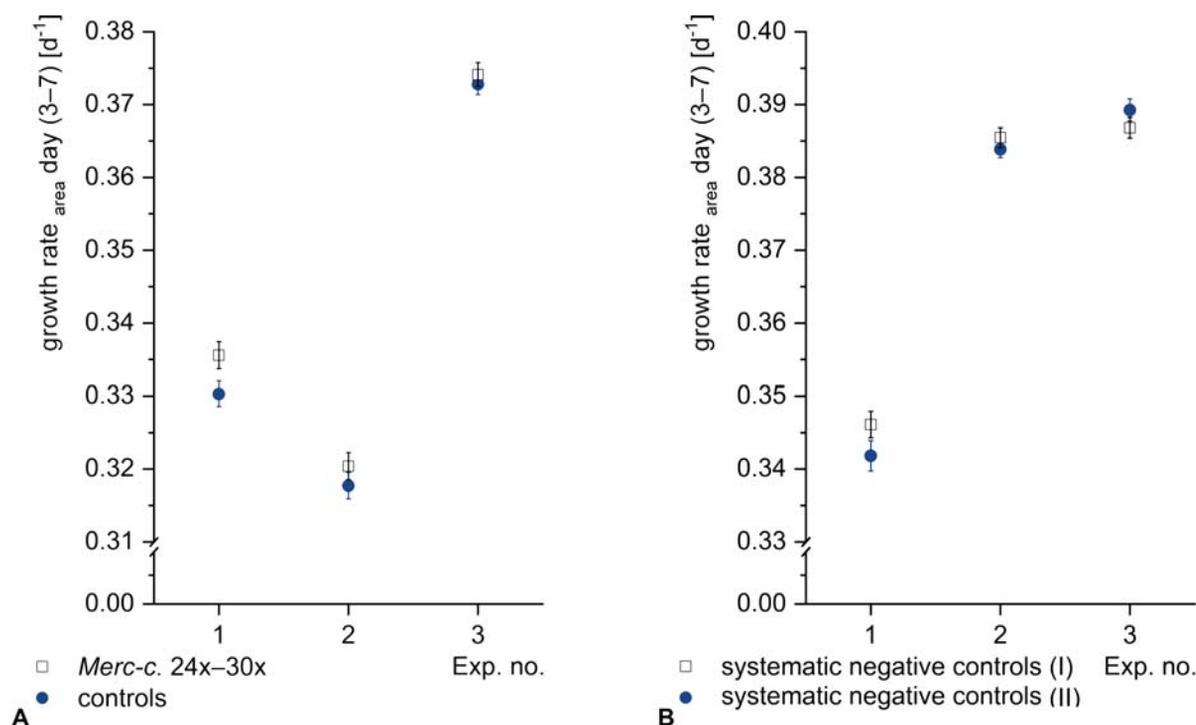
Fig. 2 Growth rate of *Lemna gibba* L. (*r*, day 3–7) [d^{-1}] (mean ± standard error) that had been stressed with 2.5 mg/L mercury(II) chloride and treated with *Merc-c.* potencies. (**A**) Data for the seven potency levels (24x–30x) were numerically pooled and compared with the pooled data for the seven control samples (three samples unsuccussed water, four samples, succussed water). The systematic negative control experiment (**B**) compared 7 × 4 beakers of unsuccussed water (systematic negative controls [I]) with another 7 × 4 beakers of unsuccussed water (systematic negative controls [II]), using the randomisation of the three *Merc-c.* experiments. The two experimental series (**A, B**) comprised three independently performed experiments each (Exp. no.).

Effects of *Merc-c.* (24x–30x, Single Potency Levels)

Duckweed growth rate data (area-related growth rates for the two time intervals) were analyzed in full two-way analysis of variance with the independent variables treatment (*n* = 9, seven potency levels and two controls) and experiment number (1–3). Results for area-related growth rate are given in ►**Table 5** (day 0–3 and 3–7) and in ►**Fig. 3A** (day 3–7). No significant effects were observed.

The SNC experiments were analyzed analogously, with randomised allocation of the beakers to the sham treatment parameters (►**Fig. 3B**). No significant effects were observed.

Discussion

Growth rate of moderately mercury-stressed duckweed was enhanced after application of potentised *Merc-c.* as measured in terms of frond area for day 3–7 (*p* < 0.05). In the first growth period of day 0–3 no significant effect was observed. Due to the inherent use of SNC experiments that did not yield any significant effect and due to additional control calculations, false-positive results can be excluded with a very high degree of certainty.

The SNC experiments revealed very small coefficients of variation for all outcome parameters measured (1.7–4.5%, ►**Table 1**). The CV decreased for the second growth period (day 3–7), an observation that accords with the hypothesis of

Table 5 ANOVA of the two experimental series (potentised test substance *Merc-c.* as well as systematic negative control [SNC] experiments) with the independent parameters experiment number (*n* = 3 independent experiments) and treatment (*n* = 9, seven potency levels [24x to 30x] and two controls [c0, c1] in the *Merc-c.* experiments, or nine samples of unpotentised controls in the SNC experiments, respectively). Measurement parameters were frond area-related growth rates for two different time intervals (day 0–3 and 3–7).

Experimental series	Effects	*p*-Values for growth rate (*r*)	
		Day 0–3	Day 3–7
Merc-c.	1: Exp. no.	<0.001	<0.001
	2: Treatment	0.686	0.175
	1/2: Interaction	0.877	0.723
SNC	1: Exp. no.	<0.001	<0.001
	2: Treatment	0.363	0.415
	1/2: Interaction	0.839	0.875

Abbreviation: ANOVA, analysis of variance.

a decreasing variation in growth for less stressed organisms (CV$_{day\ 0-3}$ > CV$_{day\ 3-7}$). Regarding variability of the measured outcome, the bioassay with stressed duckweed is superior to other model systems using stressed plants in homeopathic
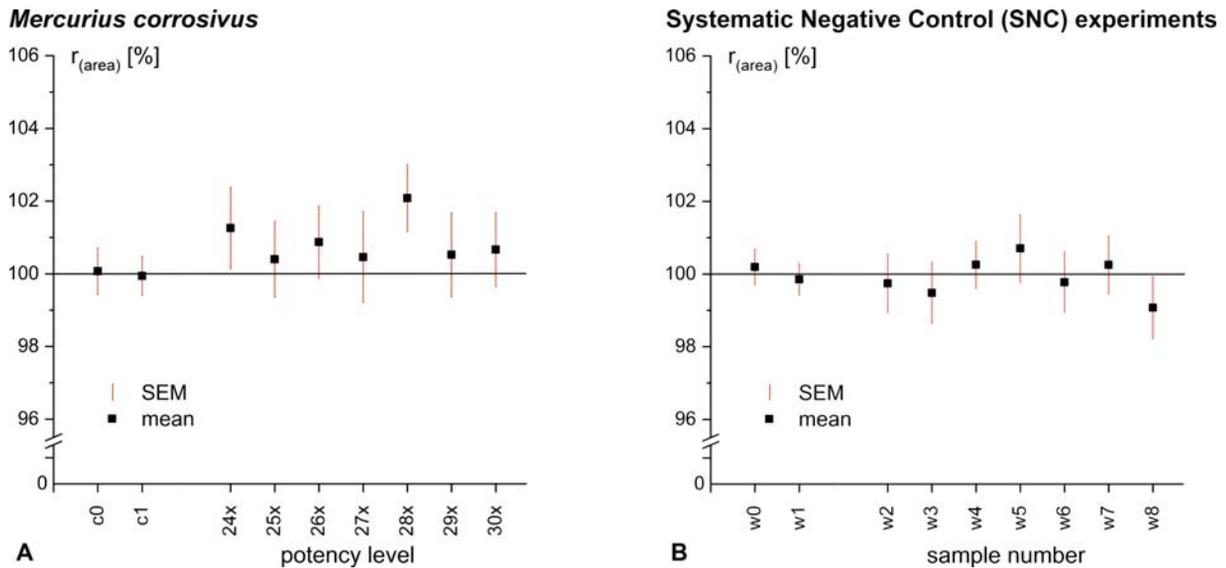
Fig. 3 (**A**) Relative area-related growth rates (*r*, day 3–7) (%) of *Lemna gibba* L. (stressed with 2.5 mg/L mercury[II] chloride) growing in different potency levels of *Merc-c.* in comparison to the corresponding water controls (unsuccussed water [c0] and succussed water [c1]). Part (**B**) shows the corresponding graph for the systematic negative control (SNC) experiments with samples of identical origin (w0–w8, unsuccussed water = dilution medium used). Mean values (dots) ± standard error (bars) for three independent experiments, respectively. Every data point for single potency levels is an average from three independent experiments with four replicates (beakers) each (*n* = 12 per data point plotted). The two data points for controls are an average from three independent experiments with 16 beakers (succussed controls c1) or 12 beakers (unsuccussed controls c0) (*n* = 48 and *n* = 36 per data point plotted). Data were normalised to the experimental mean of succussed and unsuccussed water controls (c0 + c1 = c) for each individual experiment.

basic research, since typical CVs are in the order of 10 to 80%.[15,17,19] We thus conclude that the present test system with mercury-stressed duckweed exhibited a low standard deviation.

In previous experiments, treatment of moderately arsenic-stressed duckweed with *Arsenicum album* (*Ars.*) in potency levels between 17x and 33x also yielded a growth-enhancing effect, measured in the second growth period of day 2–6. Analogously to the present dataset with *Merc-c.* preparations, no effect was observed in the first growth period of day 0–2 for *Ars.* potencies.[26] We thus conclude that the homeopathic basic research model based on arsenic-stressed duckweed treated by *Arsenicum album* potencies can be generalised to mercury-induced stress and potentised *Merc-c.*

In former experiments (Exp. Series no. 1)[21] with severely mercury-stressed duckweed *Merc-c.* potencies induced growth inhibition in the first growth period of day 0–3, whilst no effect was observed in the second growth period of day 3–7. We see these results as confirmation of our hypothesis that the growth inhibition induced by *Merc-c.* in the former experiments[21] is a consequence of the more pronounced stress in these experiments. We proposed a stress-response model[21] with five ranges: (1) low stress with no significant homeopathic effect; (2) moderate stress with a significant growth-enhancing effect of the homeopathic treatment; (3) medium stress with neutralised effects; (4) severe stress with a significant growth-inhibiting effect after homeopathic treatment; (5) very severe stress without homeopathic treatment effect (►**Fig. 4**).

According to this model, moderately stressed organisms react with growth enhancement after homeopathic treat-ment in the second growth period of day 3–7. In this period the stress is moderate (range 2) compared with a medium stress in the first growth period day 0–3 (range 3, immediately after stress application) where no homeopathic treatment effect was observed. The findings of the present experiments with moderately mercury-stressed duckweed correspond to former experiments,[26] in which potencies of *Arsenicum album* yielded a growth-enhancing effect in moderately arsenic-stressed duckweed for day 2–6 (range 2, moderate stress). The zero-treatment effect for day 0–2 in the experiments with moderately arsenic-stressed duckweed[26] would correspond to range 3 (medium stress).

We wish to point out that the abscissa in ►**Fig. 4** could be non-linear. According to the present data, a growth reduction of 12.9% for day 3–7 led to growth-enhancing effects through potentised *Merc-c* (range 2). A growth reduction of 16.1% for day 3–7 in the former trial[21] with *Merc-c.*, as well as the growth reduction during day 0–3 of 33.8% in the present trial, did not lead to significant effects (corresponding to range 3). The growth reduction during day 0–3 of 49.3% in the former trial[21] with *Merc-c.* would correspond to range 4.

We furthermore propose the hypothesis that missing or reverse effects in basic homeopathy research[12,13,32] could be explained by a relatively small range of stress levels appropriate for inducing a therapeutic effect of potentised preparations in organisms (►**Fig. 4**). Our present study and the former studies[21,26] are in line with the hypothesis.

The control calculations conducted in the present and previous experiments[21,26] indicate that the effects of potentised preparations cannot be reduced to artifacts. Furthermore, systematic errors would not lead to growth-enhancing
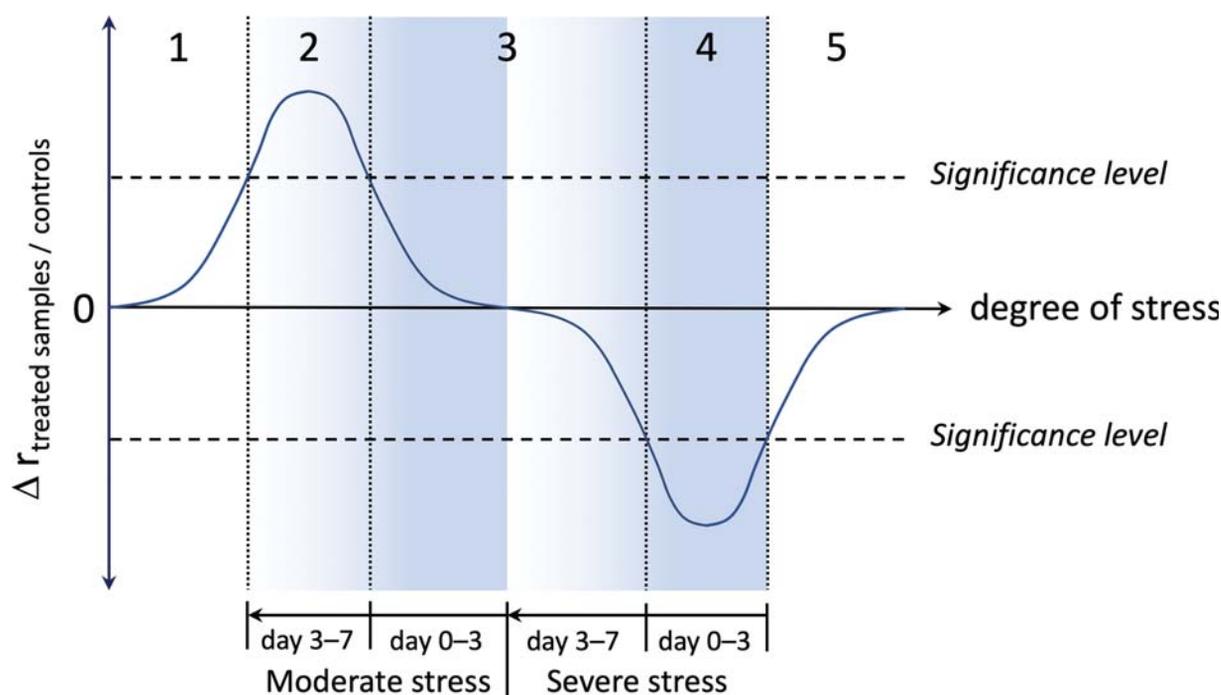
Fig. 4 Hypothesis for the homeopathic treatment response of a function of different stress levels, adapted from Jäger and colleagues 2019.[13] Diagrammatic plot (proportions not based on experimental data) of difference in growth rates between treated and untreated plants (Δr) as a function of degree of stress for *Lemna gibba* L. Five ranges are proposed: (1) low stress with no significant homeopathic treatment effect; (2) moderate stress with a significant stress-relieving effect by the homeopathic treatment; (3) medium stress with neutralised effects; (4) severe stress with a significant inhibiting effect after homeopathic treatment; (5) very severe stress without homeopathic treatment effect. As a working hypothesis we assign the aforementioned ranges 2 to 4 on the abscissa to growth phase (day 0–3, 3–7) and stress level (moderate and severe, corresponding to 2.5 and 5 mg/L mercury[II] chloride, respectively) of the mercury-stressed duckweed model system.

or growth-inhibiting effects as a function of stress level. Reverse effects controlled by the degree of stress are in favor of the notion that the duckweed bioassay with stressed organisms is a highly stable test system.

The lowest potency level in the experimental series with *Merc-c.* was 24x, corresponding to a nominal concentration of 7.5^{-27} g $HgCl_2$/L, i.e., well beyond the Avogadro limit. Significant effects from preparations beyond the Avogadro limit have also been reported for duckweed experiments with growth-enhancing effects,[26] as well as in several other biological systems.[12,13] Specific effects at these high-dilution levels, where the probability of finding any molecules of the potentised substance is extremely low, suggest informational and/or force-like (non-material) effects.

We did not observe any effect of the succussion procedure itself in this bioassay. Thus, duckweed does not seem to react to physico-chemical changes induced by the succussion of water in glass vessels (increased level of glass ions, air suspension, and dissolution, etc.).[33,34] These results are also in line with other experiments with stressed duckweed[26] and further recent investigations with various biological test systems where no significant effects of water succussion have been observed.[25,35,36]

As had been defined *a priori*, we used a numerical pool of unsuccussed and succussed water samples as control to determine the effects of *Merc-c.* 24x–30x. According to considerations published in detail elsewhere,[30] we assume this procedure to be the best approach to control unspecific, purely physico-chemical influences. The use of potentised water (at the same potency levels as the *Merc-c.* potencies used) as control would have the disadvantage that the potentised water could carry some informational content that influences growth in the model system chosen, which in turn could lead to false-negative or false-positive results. We argue that succussion only (instead of potentisation) is the most appropriate control, hypothesizing that succussion is a purely physico-chemical process that does not involve any information transfer.

Potentised medicines were observed to induce an equilibrating effect on variance in some basic research assays.[16] To test whether this phenomenon can also be observed for the present experimental series, all single experiments with *Merc-c.* (growth rate [r], day 0–3, 3–7) were analyzed by Levene's test for a difference in variance between the pooled potency levels and pooled controls. No significant differences were found.

Outlook

To further scrutinise the proposed stress-response model with five ranges of stress intensities, the experiments of this study should be repeated with different stress levels, in particular with medium stress, for which we expect neutralising effects.

For use in future research projects, the present experimental set-up might be further optimised by adjusting several experimental parameters: e.g., time of impairment in relation to time of homeopathic treatment, measurement time, growth conditions (light and temperature regimen), and modalities of application. Hitherto we avoided applying stressor and

homeopathic preparations simultaneously in the duckweed model systems, to prevent opposing effects at the same time. However, a daily addition of mercury might make it possible to keep the stress level constant over the entire test period during the application of homeopathic preparations. Such a procedure might lead to further stabilised effects of potentised preparations in the duckweed model system.

Likewise, a daily addition of homeopathic preparations may enhance the efficacy of the homeopathic treatment. Due to the lower complexity of plant organisms compared with human beings, it might be possible that the intensity of the homeopathic treatment has to be increased in plant bioassays. This procedure of iterated application of homeopathic preparations would also make it possible to change the potency level during the course of the test period if desired.

For the development of test systems to investigate questions of pharmaceutical interest (e.g., stability of homeopathic preparations against aging or external influences), restricting the range of the tested potency levels to "active" potency levels only and increasing the number of replicates per potency level could be used to increase the effect size of the test system.

A further important topic of homeopathic research is the applicability of the simile principle, which could be investigated by combining different stressors and different potentised substances. One question, for example, is whether the growth of arsenic-stressed duckweed – physiologically very similar to mercury-stressed duckweed – could be enhanced by *Merc-c.* and vice versa. Further down the line, it might be interesting to test a combination of medicines like mercury and arsenic (both heavy metals) or other potentised preparations like *Mercurius bijodatus*, in contrast to the isopathic preparation used here, *Merc-c.* (mercury(II) chloride), using both severely as well as moderately stressed duckweed.

Continuing research is needed to reveal the specific nature of the biological effects induced in duckweed. Metabolomic analysis could be supportive in two ways. First, it could serve as an additional measurement parameter for comparison between homeopathically treated and untreated duckweed. Second, the metabolomes of moderately and severely stressed duckweed could be analyzed in comparison with defined chemical pathways activated by the homeopathic treatment. Any such results might contribute to our understanding of the biological mode of action: i.e., which kind of effects homeopathic preparations induce in living organisms.

Future potential applications of this test system include testing the influence of certain pharmaceutical procedures (e.g., autoclaving, trituration vs. dilution, machine potentisation) or other external influences (e.g., heat, light, electromagnetic radiation) that might affect stability and quality of homeopathic preparations. The investigation of external influences might also help identify the physico-chemical mode of action of potentised preparations.

Conclusions

The present experimental set-up with moderately mercury-stressed *Lemna gibba* L. yielded significant growth-enhancing

effects of *Mercurius corrosivus*, compared with water controls, for the outcome parameter frond area ($p < 0.05$). These results are in contrast to the effects with severely stressed duckweed, where potentised preparations were observed to induce a growth-*inhibiting* effect ($p < 0.05$). We hypothesise that impaired duckweed responds to homeopathic preparations as a function of the stress level applied.

Highlights
- Moderately mercury-stressed *Lemna gibba* L. yielded evidence of growth-enhancing specific effects of *Merc-c.* 24x to 30x.
- This observation is complementary to previous experiments with severely mercury-stressed duckweed, in which a growth decrease was observed.
- We hypothesise that the differing results are associated with the level of stress intensity (moderate vs. severe).

Conflict of Interest
None declared.

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