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Development of a Test System for Homeopathic Preparations Using Impaired Duckweed (*Lemna gibba* L.)

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Abstract

Objectives: A bioassay with arsenic-stressed duckweed (*Lemna gibba* L.) was developed to study potentially regulative effects of homeopathic preparations. We compared potentized substances (nine different potency levels between 17 x and 33 x) with two controls (unsuccussed and succussed water) regarding their influence on number- and area-related growth rate and color of fronds (leaves). Screening included 11 potentized substances: *Arsenicum album*, gibberellic acid, nosode, arsenic(V), phosphorus, *Conchae, Acidum picrinicum, Argentum nitricum, Crotalus horridus, Hepar sulfuris*, and *Mercurius vivus naturalis*.

Design: Duckweed was stressed with arsenic(V) for 48 hours. Afterwards, plants grew in either potentized substances or water controls for 6 days. Growth rate and color of fronds were determined with a computerized image analysis system for different time intervals (days 0–2, 2–6, 0–6). A systematic negative control experiment with unsuccussed water was used to investigate the stability of the bioassay. All experiments were randomized and blinded.

Results: Arsenicum album and nosode potencies increased frond number–related growth rate compared to controls (succussed water controls or pooled water controls [succussed and unsuccussed], $p < 0.05$, t test). Regarding color classification, no effects were observed.

Conclusions: The experimental setup with *L. gibba* stressed by arsenic(V) provides a valuable tool to investigate regulative effects of potentized substances. In order to verify the effects of *Arsenicum album* and nosode potencies, further independent replication experiments are necessary.

Introduction

Identification of the active principle of homeopathic remedies prepared by logarithmically diluting and succussing the original substance is a major scientific issue for homeopathy.[1–3] In high dilutions (>12c or 24 x , respectively), the probability for even a single molecule of the mother tincture to be present is near zero. Thus, specific effects of high potencies are considered to be implausible according to the current scientific paradigm.[4,5] Quantitative meta-analyses of randomized clinical trials covering all kind of indications yield inconclusive evidence for the efficacy of homeopathic remedies, and seem to be dependent on the inclusion criteria applied.[6–10] When restricted to specific medical conditions, quantitative meta-analyses of randomized controlled trials in the majority of cases reported significant homeopathic remedy effects compared to placebo.[11–16] Thus, it seems that—at least in certain cases—the dilution medium may adopt specific properties related to the mother tincture potentized, even without any molecules of the latter being present.

Assuming that homeopathic remedies induce specific effects, there is considerable interest in revealing their mode of action as well as the consequences of certain pharmaceutical procedures (e.g., autoclavation) or other external influences (e.g., electromagnetic radiation) that might affect the efficacy of homeopathic preparations. However, identification of adequate test systems and apt methods for homeopathic basic research yielding reproducible effects of homeopathic remedies is still an unsolved issue.[17,18]

Based on the assumption that a characteristic feature of homeopathic preparations is to induce equilibrating

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(regulative) effects, test systems with impaired organisms are to be expected to yield larger effect sizes compared to test systems using healthy organisms. However, test systems with impaired organisms usually exhibit considerable increase of variance.[19] Hence, major attention has to be given to a high degree of standardization to achieve a standard deviation as low as possible.

In this investigation, a botanical test system was used. Botanical systems are usually fast and simple, allowing large numbers of experimental replications, eliminating disadvantages such as the placebo effect or ethical concerns, and they have been shown to react to potentized substances[20,21] also after abiotic stress.[22–26] Duckweeds have often been used in bioassays as standardized research organisms[27–29] due to their small size and rapid, predominantly vegetative reproduction forming genetically uniform clones as well as their high sensitivity to organic and inorganic substances including arsenic.[30–33] Furthermore, unimpaired duckweed recently has been successfully introduced in homeopathic basic research.[34,35]

To develop a method with impaired duckweed, an appropriate stressor had to be found based on experimental preselection. Furthermore, several experimental parameters (e.g., degree of damage) were optimized using an isopathic experimental approach before screening several potentized test substances to alleviate the stress induced.

Materials and Methods

Development of the experimental setup

After preliminary tests with different stressors, variance of duckweed growth rates was compared in detail for three stressors: ultraviolet-B radiation, copper sulfate, and arsenic. Arsenic was finally chosen as stressor in our assays due to its small intra- and interexperimental variance. Subsequently, several combinations of experimental parameters (e.g., degree of damage, day–night period, time, and duration of impairing) as well as different experimental outcome parameters (e.g., frond number and frond area–related growth rates, color classification, dry weight) were tested in an isopathic approach with arsenic(V) using potency levels from 14 x to 45 x, in order to determine optimal experimental settings.

Preparation of homeopathic dilutions and controls

The choice of homeopathic remedies included in the screening experiments reported here (Table 1) was based on several approaches. Besides investigating an isopathic application, substances to be tested in potentized form were chosen according to related chemical properties, plant metabolism, previous experiments,[35] and in cooperation with physicians.

Before each experiment, all potentization vessels (see below) were thoroughly rinsed with de-ionized water ($<0.5\,\mu S/cm$, Christ Milistil P-24, Christ-Aqua-Ecolife, Aesch, Switzerland), three times before and three times immediately after agitating for 20 minutes (Universalschüttler SM-30-B, Edmund Bühler GmbH, Hechingen, Germany) with de-ionized water. Then vessels were dried at 90°C for 70 minutes. Water for potentization and controls was distilled (Büchi, Fontavapor-250, Flawil, Switzerland) from tap water (Institution Arlesheim, Switzerland) and autoclaved (Getinge AB-Typ-GE-406, Sweden) before use. All test solutions for one experiment (potencies as well as controls) were freshly prepared from the same batch of distilled water. During the experiments, the contact of water and plastic material was circumvented to avoid dissolving of toxic substances in water.[36–39]

For preparation of the nosode, duckweed grew for 48 hours in 2 L modified Steinberg medium (moStM) comprising 158 mg/L arsenic(V). Duckweed was cut into small pieces, mixed with 85 mL distilled water and 15 mL ethanol (94%, Alcosuisse-S15-sekunda, Schachen, Switzerland), and agitated for 2 hours (Turbula T2 C, Willy A. Bachofen AG, Basel, Switzerland) in an Erlenmeyer flask of Duran® glass (250 mL, Schott, Mainz, Germany). After maceration at 20°C and diffused light for 21 days, the extract was filtered (Macherey-Nagel, MN-619-eh ¼ diameter 185 mm, Germany) and stored at 4°C for 12 days.

Arsenic(V), nosode, and *Conchae* were potentized in distilled water from 1 x to 33 x (Table 1). Gibberellic acid was potentized in acetone (AppliChem-A2300 Darmstadt, Germany) to 1 x, then further potentized in distilled water. All other homeopathic remedies were obtained from pharmaceutical companies in the lowest potency available (1 x to 10 x) and further potentized in distilled water. All homeo-

Table 1. Substances Included in the Screening Experiments

Substance, empirical formula	*Category*	*Source*	*Concentration of potency stock preparation*
Arsenic(III) (*Arsenicum album*; As_2O_3)	Metal	Weleda, Arlesheim, Switzerland	5 x, trituration
Gibberellic acid; $C_{19}H_{22}O_6$	Plant hormone	Sigma-Aldrich, Buchs, Switzerland	10 mg/1000 mL
Nosode	Plant digestion	Own laboratory	100 g/900 mL
Arsenic(V) (sodium dibasic arsenate 7-hydrate; $AsHNa_2O_4 \times 7H_2O$)	Metal	Sigma-Aldrich, Buchs, Switzerland	100 g/900 mL
Phosphorus	Nutrient	Weleda, Arlesheim, Switzerland	3 x, dilution
Conchae	Oyster shell	Weleda, Arlesheim, Switzerland	100% powder
Acidum picrinicum	Explosive agent	Remedia, Eisenstadt, Austria	3 x, dilution
Argentum nitricum	Metal	Weleda, Arlesheim, Switzerland	10 x, trituration
Crotalus horridus	Venom	DHU, Karlsruhe, Germany	6 x, dilution
Hepar sulfuris	Oyster shell and flower of sulfur	Weleda, Arlesheim, Switzerland	1 x, trituration
Mercurius vivus naturalis	Metal	Weleda, Arlesheim, Switzerland	3 x, trituration

pathic potencies were prepared according to the multiple glass method between 6:00 and 9:00 AM on the day of the experiment. All experiments were carried out in laboratories at the Research Institute of Organic Agriculture (FiBL), Frick, Switzerland between February and August 2009.

For the potentization process, Erlenmeyer flasks of Duran® glass (<6 x: 250 mL, > 5 x: 500 mL, Schott, Mainz, Germany) were used. Fifteen milliliters (15 mL) of potency stock solution was added to 135 mL distilled water. Then the Erlenmeyer flask was agitated once upside-down to generate a vortex. After slowing of the vortex, the flask was shaken a second time, producing a chaotic agitation in water. These two steps were repeated 10 times. For the next potency level, 15 mL of this solution was added to the next potentization vessel containing 135 mL distilled water and agitated in the same manner. With potency level 7 x, flask size was changed from 250 to 500 mL, and the filling volume raised to 350 mL; thus, 35 mL of the former potency level was added to 315 mL of distilled water. This process of successive 10-fold dilution steps and vigorously shaking proceeded until the potency step 33 x was accomplished.

Four (4) potentization vessels containing 350 mL of distilled water each serve as unsuccussed water controls (c0). Another five vessels were succussed once exactly as described before for one potency step (control c1, corresponding to water potentized to 1 x).

In the screening experiments, nine potency levels (17 x, 18 x, 21 x, 22 x, 23 x, 24 x, 28 x, 30 x, 33 x) of a given test substance as well as nine control samples (four samples unsuccussed [c0] and five samples succussed [c1] water) were investigated. After preparation, all test solutions were randomized and coded (blinded) by a person not involved in the experiments by random assignment of double letter code from a predefined list.

Duckweed bioassay

Duckweed (*Lemna gibba L.*, clone no. 9352) had been obtained from a laboratory culture of Aachen Technical University, Germany. Cultures were grown in moStM according to a standard of the International Organization for Standardization[29] (all ingredients for moStM were obtained from Fluka, Buchs, Switzerland). Stock solutions nos. 1–3, 8, and 9 were individually prepared with autoclaved distilled water (Büchi, Fontavapor-250, Flawil, Switzerland), while nos. 4–7 were pooled. Prior to the experiments, the final medium (pH 5.8 ± 0.2) was freshly prepared with autoclaved distilled water.

Previous to the experiments, *L. gibba* from axenic (pure) stock cultures on 50 mL solid moStM with 1% (w/v) dextrose glucose, (Becton, Dickinson and Company, Sparks) and 1% (w/v) bacteriologic agar No.1 (Oxoid, Basingstoke, UK) added were adapted to 150 mL of liquid autoclaved moStM in Erlenmeyer flasks for at least 4 weeks, and in larger glass vessels containing 2 L of moStM for another 4 weeks in order to acclimatize and get large amounts of plants. The medium was changed weekly to achieve a rapid growth near to exponential (i.e., growth was not restricted, e.g., due to space limitations or nutrient restrictions).

The last change of moStM was 48 hours before the start of the experiment. Plants were transferred to a vessel containing 2 L freshly prepared moStM to ensure identical nutrient concentration when adding 158 mg/L arsenic(V) ($AsHNa_2O_4 \times 7H_2O$, Sigma-Aldrich, Buchs, Switzerland). Twenty-four (24) hours before the start of the experiment, fronds damaged too strongly were removed from the vessel. After 48 hours, arsenic-treated duckweed exhibited an area-related growth rate of approximately 44% compared to duckweed growing without arsenic ($r_{\text{with arsenic}} = 0.16\ d^{-1}$, $r_{\text{without arsenic}} = 0.36\ d^{-1}$).

On the day of the experiment, plants without visible lesions, chlorosis, or necrosis were selected from the vessel ($\approx 1.5\%$) and sorted in three groups according to number of fronds (three fronds per colony) and similar size. For each experiment, 50 mL of moStM was poured (Bottletop dispensing head, 50 mL, Wertheim, Germany) in 90 beakers each (150 mL, SIMAX,® Kavalier, Sázava, Czech Republic, Fig. 1). Then 50 mL of 18 coded samples (potencies or controls) in five replicates each was added to the 90 beakers. Hence, each screening experiment was conducted with nine different potency levels of one test substance in five replicates each as well as four groups of five beakers of unsuccussed (c0) and five groups of five beakers of succussed (c1) water controls (i.e., 45 beakers with potencies and 45 beakers with controls). The 18 samples were added in a blocked randomization scheme. The latter had been successfully tested in pre-investigations with unsuccussed water only to be sure that the spatial arrangement does not pro-

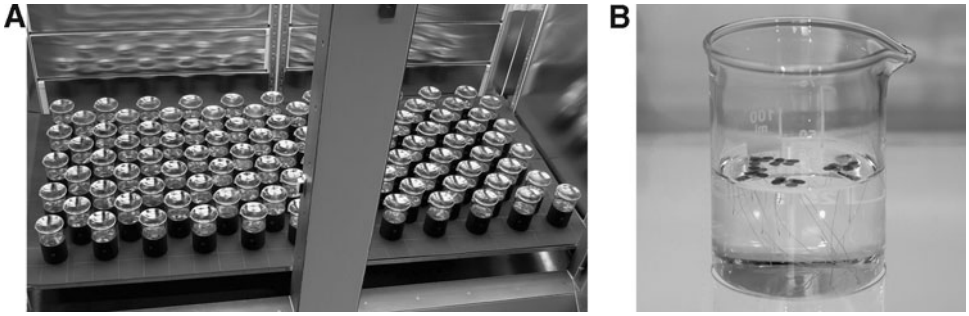


FIG. 1. **A.** Experimental setup of a single experiment in the growth chamber (100 beakers with *Lemna gibba*). For every experimental parameter ($n = 20$ in total, $n = 18$ letter-coded samples and two additional open controls, one with unimpaired duckweed and one with duckweed, impaired during the entire experimental period; the latter two controls were not used for the statistical evaluation), five replicates were used and allocated in a fixed blocked randomization scheme. **B.** Single beaker with duckweed.

duce false-positive results. Afterwards, the sorted duckweed colonies were carefully placed into the beakers, so that every beaker contained the same number of fronds of similar size (nine fronds) at the beginning of the experiment.

Material that was used for different experimental conditions (bottletop dispensing head, *Lemna* transfer wire hook) was carefully rinsed three times with de-ionized water in order to minimize cross-contamination.

Frond area, frond number, and percentage of color classes were measured in every beaker using an image processing system (Scanalyzer, duckweed analytic software, version 4, LemnaTec, Aachen, Germany) consisting of video camera, computer, and adapted software. On days 0, 2, and 6 of each experiment, a digital image was obtained from every beaker (Fig. 2). For each recorded image, the quality of the automatic image analysis was checked and corrected by hand if necessary.

Before beakers were set up in a plant growth chamber (Percival AR-75L, Iowa) they were wrapped in black paper up to the surface of the test solution and put on black paper in order to eliminate any diffused light from the side or the bottom (Fig. 1). Underlayment was foam (Ethafoam, Fritz Nauer AG, Wolfhausen, Switzerland). Each beaker was covered with a watch glass to avoid excessive evaporation or accidental contamination.

Duckweed cultures grew in the plant growth chamber under constant (24 hours) illumination with fluorescent light ($137 \pm 0.6\,\mu$mol photons m^{-2} s^{-1} photosynthetically active radiation (PAR), F32 T8/TL 741, Philips, Switzerland). The mean air temperature was $21.5^{\circ}C \pm 0.5^{\circ}C$, the mean temperature of the Steinberg medium was $22.6^{\circ}C \pm 0.3^{\circ}C$ (Endotherm, Dornach, Switzerland), and the mean relative humidity was $62\% \pm 7\%$ (Ebro EBI-20-TH, Ingolstadt, Germany). The growth rate of 11 experiments (mean of 45 controls per experiment) and the systematic negative control experiment (mean of 45 pseudocontrols per experiment) over the complete period of experiments (February–August 2009) averages $r_{(area)} = 0.42 \pm 0.02$ d^{-1}.

Based on the measurements of frond area and frond number, the average growth rate per day (r) was calculated for three test periods (days 0–2, days 2–6, and days 0–6) according to the equation: $r = (\ln(x_{t2}) - \ln(x_{t1}))/(t_2 - t_1)$, where x_{t1} is the value of observation parameter at day t_1, x_{t2} is the value of observation parameter at day t_2, and $t_2 - t_1$ is the time period between x_{t1} and x_{t2} in days. Percentages of three measured color classes (dark green, verdure, light green; shown in black and white) of frond area were calculated by difference in means ($\Delta c_{(\text{color class})} = c_{\text{day 6 (color class)}} - c_{\text{day 0 (color class)}}$) for days 0–6 only.

After every experiment, all beakers were cleaned first with a hot tap water shower and then using a brush under running hot tap water, and then cleaned in a dishwasher (Renggli, Rotkreuz, Switzerland). Afterwards beakers were given an acid bath (3% HNO_3) for 24 hours, rinsed under tap water, cleaned in a dishwasher, thoroughly rinsed under running deionized water, and finally dried at 90°C. Beakers were reused in subsequent experiments in randomized order.

Statistical analysis

A comparison of growth rate data ($r_{(area)}$ and $r_{(num)}$) and color classification data ($\Delta c_{(\text{color class})}$) between pooled potencies and pooled water controls (successed and unsuccessed) was effectuated by means of *t*-tests for

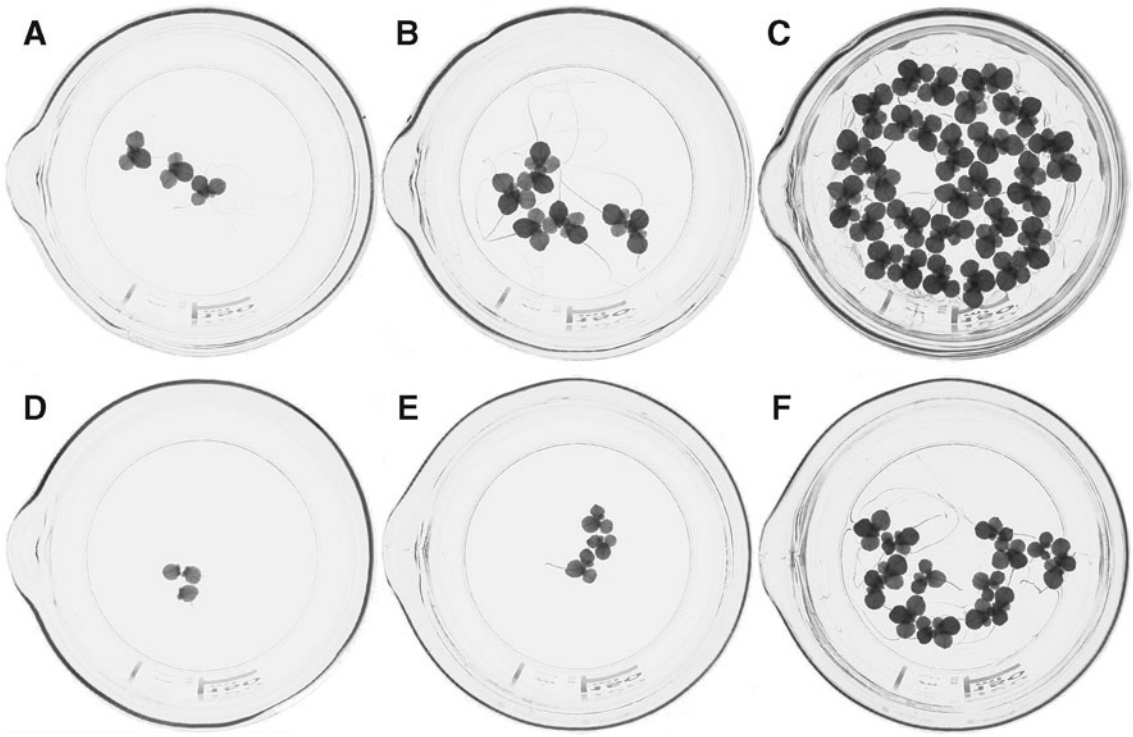


FIG. 2. Unimpaired *Lemna gibba* (day 0 **[A],** day 2 **[B],** and day 6 **[C]**) and arsenic(V)-impaired *L. gibba* (day 0 **[D],** day 2 **[E],** and day 6 **[F]**) in one representative beaker each.

Table 2. Coefficient of Variation (CV) for Each Outcome Parameter in the Systematic Negative Control Experiment, Based on Mean Values of 18 Groups of 5 Replicates (Total 90 Beakers)

	Growth rate $r_{(area)}$			*Growth rate* $r_{(number)}$			$\Delta_{(color\ class)}$ *Days 0–6*		
	Days 0–2	*Days 2–6*	*Days 0–6*	*Days 0–2*	*Days 2–6*	*Days 0–6*	*Dark green*	*Verdure*	*Light green*
CV (%)	3.3	1.2	1.3	2.7	2.3	1.4	6.7	6.2	10.7

independent samples. Regarding a possible succussion effect, data of the unsuccussed (c0) and succussed water controls (c1) of all 11 screening experiments were also compared using the *t*-test. Data distribution had been evaluated graphically by quantile–quantile plots. All analyses were made using the software STATISTICA Version 6 (Stat Soft, Tulsa, OK).

Results

All experiments were documented by a total of 3240 digital beaker images (12 experiments × 3 measurements × 90 beakers) corresponding to 38,880 data points (3 + 3 for frond area and number, 6 for three color classifications). Data from three measurements are lacking due to software failures. All data entered statistical analysis (extreme values were not eliminated).

Variability of the bioassay

Data from the systematic negative control experiment (90 identically treated beakers with unsuccussed water) were used to estimate the variability of the bioassay. We grouped the data of the 90 beakers into 18 groups of five replicates (beakers) and calculated mean values for these 18 subgroups for the following outcome parameters: frond area- and frond number–related specific growth rate (days 0–2, 2–6, 0–6 each) as well as color classification (days 0–6 only). Based on these 18 mean values, the coefficient of variation was calculated for each outcome parameter (Table 2).

When the systematic negative control experiment was evaluated in the same way as the experiments with homeopathic preparations, no significant differences were observed ($p > 0.24$ for all outcome parameters, see below). Thus, the experimental setup was considered to be stable.

Succussion effect

Unsuccussed (c0) and succussed (c1) water controls from all 11 experiments that included potentized substances (no water control experiment) were compared to detect any effects of the succussion procedure itself. Six (6) of 99 *p*-values in total (9 outcome parameters × 11 experiments) were below $p < 0.05$; none were below 0.01 (Table 3).

Screening experiments

The effect of 11 potentized substances on impaired *L. gibba* was investigated by comparing all nine tested decimal potency levels pooled with the water controls regarding frond area- and number-related growth rates as well as three different color classes of duckweed. Since there were no differences between succussed (c1) and unsuccussed (c0) controls, one may pool the data from both types of controls and compare the homeopathic preparations to this pool of controls in order to increase statistical power (Table 4). Alternatively, one may compare the potencies with the succussed controls only (Table 5). In the first approach, six of 99 *p*-values in total were below $p < 0.05$, and two were below 0.01; in the second approach, 10 *p*-values were below $p < 0.05$, and four were below 0.01.

Table 3. Screening Experiments with Impaired *Lemna gibba:* Comparison (*t*-Test) of Unsuccussed (c0) and Succussed Controls (c1) by Two Growth Parameters in Three Time Intervals and Three Color Classes, for All Experimental Runs

	p-*Values for growth rate* $r_{(area)}$			p-*Values for growth rate* $r_{(number)}$			p-*Values for* $\Delta_{(color\ class)}$ *Days 0–6*		
Experimental run	*Days 0–2*	*Days 2–6*	*Days 0–6*	*Days 0–2*	*Days 2–6*	*Days 0–6*	*Dark green*	*Verdure*	*Light green*
Arsenic(V)	0.209 +	0.335 +	0.244 +	0.625 −	0.511 +	0.700 +	0.985 +	0.978 +	**0.016** −
Hepar sulfuris	0.160 +	0.219 −	0.786 −	0.305 +	**0.047** −	0.262 −	0.842 +	0.774 −	0.843 −
Mercurius	0.863 +	0.778 +	0.790 +	0.823 +	0.328 +	0.197 +	0.715 −	0.993 −	**0.036** +
Phosphors	0.485 +	0.693 +	0.571 +	0.823 −	0.911 +	0.971 −	0.807 −	0.803 +	0.388 +
Conchae	0.287 +	0.475 −	0.968 +	0.729 −	0.126 −	0.080 −	0.781 +	0.837 −	0.488 −
Acidum picrinum	0.360 −	**0.038** −	0.760 −	0.492 −	0.226 −	0.071 −	0.884 +	0.925 −	0.838 +
Argentum nitricum	0.234 −	0.162 −	0.117 −	0.108 +	0.076 −	0.679 +	0.649 +	0.430 +	0.810 +
Crotalus horridus	0.060 −	0.277 +	0.921 −	1.000 =	0.909 −	0.909 −	0.817 +	0.941 −	0.546 −
Arsenicum album	0.900 +	0.124 −	0.362 −	0.972 +	0.329 −	0.269 −	0.995 +	0.829 +	0.911 +
Nosode	0.535 −	0.248 −	0.312 +	0.730 +	0.700 −	0.715 −	0.121 −	0.150 +	0.570 +
Gibberellic acid	0.542 −	0.368 −	0.376 −	0.207 +	**0.031** −	0.479 −	**0.031** +	0.110 −	0.188 −

p-Values of *t*-tests for independent samples; significant values ($p < 0.05$) are bold; +, increased growth rate of succussed controls; −, decreased growth rate of succussed controls; =, identical growth rate of succussed and unsuccussed controls.

Table 4. Statistical Analysis of the Systematic Negative Control Experiment (SNC) and the Screening Experiments with Impaired *Lemna gibba*: Comparison (*t*-Test) of Pooled Controls (c0, c1) and Pooled Potencies (17 x, 18 x, 21 x, 22 x, 23 x, 24 x, 28 x, 30 x, 33 x) for Two Growth Parameters in the Three Time Intervals and Three Color Classes, for All Potentized Substances Tested

	p-*Values for growth rate* $r_{(area)}$						p-*Values for growth rate* $r_{(number)}$						p-*Values for* $\Delta_{(color\ class)}$ *Days 0–6*					
Substances	*Days 0–2*		*Days 2–6*		*Days 0–6*		*Days 0–2*		*Days 2–6*		*Days 0–6*		*Dark green*		*Verdure*		*Light green*	
Arsenic(V)	0.640	+	0.627	–	0.958	+	0.588	–	0.260	+	0.413	+	0.892	–	0.199	–	0.668	–
Hepar sulfuris	0.325	+	0.965	+	0.659	+	0.279	+	1.000	=	0.392	+	0.423	–	0.408	+	0.979	–
Mercurius	0.416	+	0.447	+	0.405	+	0.148	+	0.688	–	0.260	+	0.389	–	0.951	+	0.470	–
Phosphors	0.714	–	0.637	+	0.540	+	0.540	+	0.835	–	0.808	+	0.207	–	0.136	+	0.643	–
Conchae	0.543	+	0.506	+	0.479	+	0.955	+	0.835	+	0.804	+	0.856	–	0.965	–	0.407	+
Acidum picrinum	0.211	–	0.217	+	0.798	+	0.235	+	0.486	+	0.133	+	0.918	–	0.971	–	0.555	+
Argentum nitricum	0.222	+	0.086	+	0.094	+	0.058	+	0.198	–	0.475	+	0.110	–	0.705	–	0.084	–
Crotalus horridus	0.360	–	0.966	–	0.944	–	0.080	–	0.775	+	1.000	=	0.643	–	0.693	+	0.656	+
Arsenicum album	0.261	+	0.061	+	0.086	+	0.923	+	**0.018**	+	**0.002**	+	0.867	+	0.629	–	0.503	+
Nosode	0.422	+	**0.015**	+	0.051	+	0.082	+	**0.023**	+	**0.001**	+	0.883	–	0.927	+	0.942	+
Gibberellic acid	0.705	–	0.365	–	0.435	–	0.712	+	**0.043**	–	0.095	–	0.274	+	0.377	–	0.658	–
SNC	0.265	+	0.244	–	0.825	+	0.956	+	0.740	–	0.802	–	0.416	+	0.540	+	0.556	–

p-Values of *t* tests for independent samples; significant values ($p < 0.05$) are bold; +, potencies increased growth rate (significant values: increase between 1.4% and 3.5%); –, potencies decreased growth rate (significant value: decrease of 1.9%); =, no effect on growth rate.

Formally, significant effects were observed for *Arsenicum album*, nosode, gibberellic acid, and *Argentum nitricum* for the observation periods 2–6 and 0–6 days. Comparing both evaluation approaches (pooled versus succussed controls, cf. Tables 4 and 5), consistent results were obtained for two test substances (*A. album* and nosode); both stimulated duckweed growth (Fig. 3). No effects were observed for the observation period 0–2 days, meaning that effects of the homeopathic preparations manifested mainly in newly formed fronds (leaves), rather than in old fronds.

Discussion

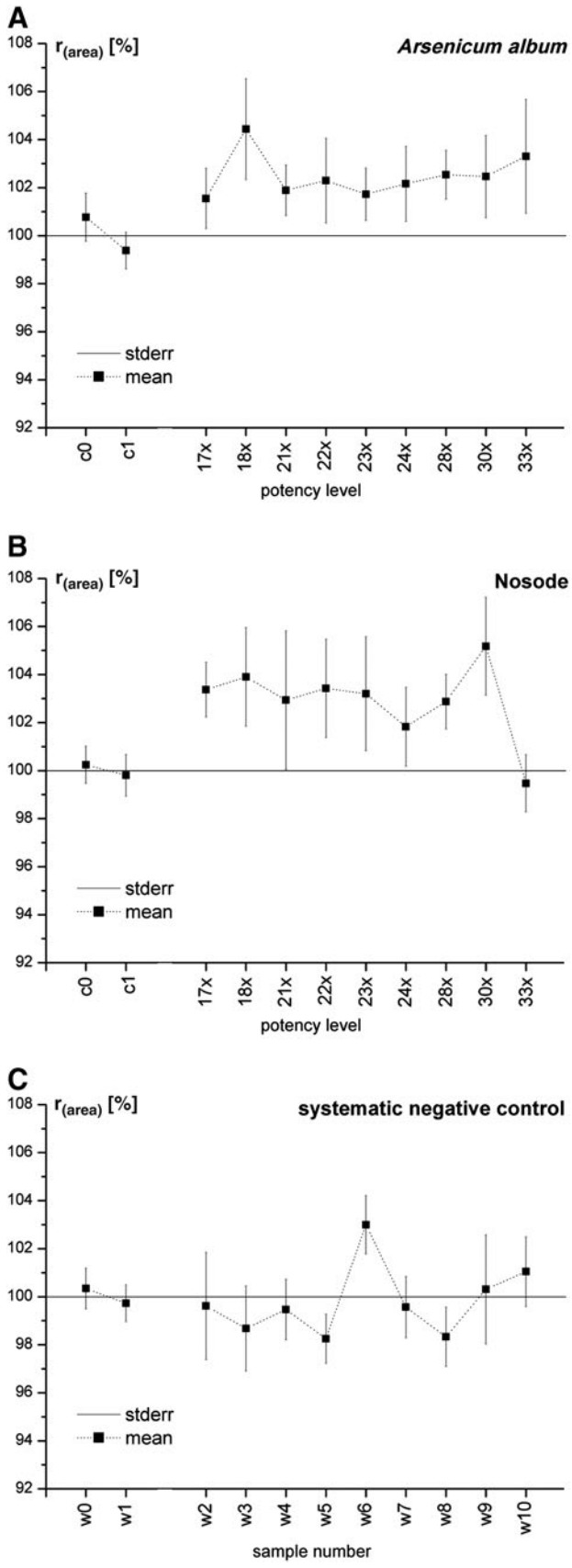
Arsenic(V)-impaired duckweed was evaluated as test organism for homeopathic remedies. The test system exhibited very small coefficients of variation, as low as 1.3% for $r_{(area)}$ days 0–6. In randomized and blinded screening experiments, 11 different potentized substances were compared with succussed and unsuccussed water controls. Of the substances tested, *A. album* and nosode potencies increased the growth rate of duckweed.

The development of an experimental setup with impaired organisms posed special challenges. A compromise had to be found for the concentration of arsenic, between a good measurable toxic effect and a vitality level ensuring sufficient self-healing power of organisms. Furthermore, arsenic concentration and consequently the degree of plant damage is positively correlated with standard deviation.[19,40] Hence, the test system had to be stabilized (e.g., by establishing a homogeneous light field in growth chamber and careful selec-

Table 5. Statistical Analysis of the Systematic Negative Control Experiment (SNC) and the Screening Experiments with Impaired *Lemna gibba:* Comparison (*t*-Test) of Succussed Controls (c1) and Pooled Potencies (17 x, 18 x, 21 x, 22 x, 23 x, 24 x, 28 x, 30 x, 33 x) for Two Growth Parameters in the Three Time Intervals and Three Color Classes, for All Potentized Substances Tested

	p-*Values for growth rate* $r_{(area)}$						p-*Values for growth rate* $r_{(number)}$						p-*Values for* $\Delta_{(color\ class)}$ *Days 0–6*					
Substances	*Days 0–2*		*Days 2–6*		*Days 0–6*		*Days 0–2*		*Days 2–6*		*Days 0–6*		*Dark green*		*Verdure*		*Light green*	
Arsenic(V)	0.786	–	0.336	–	0.480	–	0.837	–	0.560	+	0.629	+	0.910	–	0.276	+	0.425	–
Hepar sulfuris	0.968	+	0.479	+	0.605	+	0.730	+	0.294	+	0.210	+	0.439	–	0.395	–	0.922	+
Mercurius	0.561	+	0.633	+	0.585	+	0.284	+	0.458	–	0.807	+	0.604	–	0.956	–	0.079	–
Phosphors	0.472	–	0.846	+	0.869	–	0.542	+	0.816	–	0.804	+	0.363	–	0.272	–	0.414	+
Conchae	0.960	+	0.308	+	0.506	+	0.834	+	0.285	+	0.225	+	0.759	–	0.940	–	0.236	–
Acidum picrinum	0.656	–	0.362	+	0.644	+	0.790	–	0.141	+	0.127	+	0.862	–	0.867	–	0.782	+
Argentum nitricum	0.071	+	**0.043**	+	**0.036**	+	0.491	+	0.814	–	0.698	–	0.119	–	0.420	+	0.123	+
Crotalus horridus	0.884	+	0.557	–	0.756	–	0.192	–	0.650	+	0.830	+	0.580	–	0.680	–	0.374	+
Arsenicum album	0.257	+	**0.009**	+	**0.031**	+	0.949	+	**0.012**	+	**0.001**	+	0.892	+	0.611	+	0.605	–
Nosode	0.273	+	**0.011**	+	**0.028**	+	0.630	+	**0.007**	+	**0.004**	+	0.580	+	0.581	+	0.879	+
Gibberellic acid	0.976	–	0.781	–	0.838	–	0.646	–	0.449	–	0.317	–	0.949	–	0.989	+	0.796	–
SNC	0.442	+	0.245	–	0.935	–	0.636	+	0.794	–	0.928	+	0.274	+	0.368	+	0.315	–

p-Values of *t*-tests for independent samples; significant values ($p < 0.05$) are bold; +, potencies increased growth rate (significant values: increase between 2.0% and 4.4%); –, potencies decreased growth rate.



tion of duckweed plants after the arsenic stress period) without losing the sensitivity of the system toward homeopathic treatment. These considerations also influenced the choice of the arsenic species used as stressor. The dissolved species arsenic(III) and arsenic(V) are absorbed from plants in a different way and in varying amounts. Furthermore, the biologic effects on plants are different. Arsenic(III) that was used in previous studies[40] exhibits low stability under changing redox potential, pH, and light conditions.[41,42] Therefore, the current study used arsenic(V) to avoid an undefined state of oxidation, to stabilize the system and to reduce the coefficient of variation.

Arsenic(V) induces growth inhibition, chlorosis, and necrosis in plants. Several biochemical mechanisms (e.g., active exclusion) may be activated in plants to induce protection from arsenic(V) impairment. Which of these mechanisms were upregulated by the homeopathic preparations remains an interesting issue for further investigations. It is obvious that the results of this investigation cannot be directly extrapolated to humans. Further basic research may help to explain the protective effects observed in studies with human subjects.[43–45]

Interestingly, potentized arsenic(V) did not show any protective effect in this screening, in contrast to potentized *A. album*, which chemically corresponds to arsenic(III). The arsenic(V) potencies were prepared directly as aqueous potencies starting from the liquid stock solution (dissolved $AsHNa_2O_4 \times 7H_2O$) that was used for the impairment of duckweed, whereas *A. album* potencies were prepared based on a 5× trituration of As_2O_3 in lactose. It may be interesting to determine the reasons for the differing results in future experiments.

Strengths of the newly developed experimental setup include the use of genetically uniform duckweed plants with rapid reproduction, the use of chemically stable arsenic(V) as stressor, the low coefficient of variation, the comparably high effect size (corresponding to significant effects even in single experiments), the thorough documentation of the experiment by digitally stored pictures, and the automated image analysis yielding objective values for duckweed frond size. Limitations or disadvantages of the experimental setup consist of the use of a comparatively expensive growth chamber and commercial image analysis system, the need for a careful manual selection of duckweed plants after arsenic treatment, and the production of potentially ecological

FIG. 3. Number-related average specific growth rates ($r_{(number)}$ days 0–6) [%] of *Lemna gibba* growing in different potency levels of two test substances **[A, B]** or corresponding water controls [c0 and c1]). Part **(C)** shows the corresponding graph for the pure water control experiment (systematic negative control) with samples of identical origin (unsuccussed water = dilution medium used). Mean values (dots) ± standard error (stderr) (bars) for five beakers, respectively. Every data point for potencies is an average from five replicates (beakers) each. The two data points for the controls are an average from 25 (succussed control) or 20 (unsuccussed control) replicates (beakers) each. Data were normalized to the mean of succussed and unsuccussed water controls (c0 + c1). Lines connecting data points are no interpolations.

harmful waste that has to be disposed by specialized procedures.

Possible future applications of this test system can be seen in empirically documenting reproducible specific effects of highly diluted homeopathic remedies, in determining the corresponding mode of action, and in testing the stability of homeopathic preparations against external influences. It is currently unknown whether certain pharmaceutical procedures (e.g., autoclavation, sterile filtration) or other external influences (such as heat, light, and electromagnetic radiation) hamper the efficacy of homeopathic preparations. Preclinical test systems might help to identify critical procedures.

Conclusions

The present experimental setup with arsenic-impaired *L. gibba* is a feasible and promising tool to document and to investigate effects of potentized substances. To further validate this test system, it will be necessary to verify the observed effects of selected test substances in more replication experiments. Additionally, more systematic negative control experiments should be performed in order to thoroughly document the stability of the experimental setup.

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Disclosure Statement

No competing financial interests exist.

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