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Original Research Article

Multidimensional Outcome Parameters in a Cress Seedling—$CuCl_2$ Crystallization Assay to Corroborate Specific Effects of *Stannum metallicum* 30x Compared to Lactose 30x

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Homeopathy

Abstract

Background Previously we developed a test system which yielded highly significant evidence for specific effects of a *Stannum metallicum* 30x preparation in a multi-center replication trial. This test system is based on cress seed germination in homeopathic or control samples, $CuCl_2$ crystallization of the cress extract, and subsequent digital textural image analysis of the resulting crystallization patterns.

Objectives The current study aimed to investigate whether three novel outcome parameters could further corroborate and possibly characterize the specific effects of *Stannum metallicum* 30x.

Methods To this end, (1) cress seedling length, (2) a second texture analysis parameter, entropy and (3) the local connected fractal dimension (LCFD) of crystallization patterns as a measure of complexity were considered. The stability of the experimental setup was monitored throughout the entire investigation with systematic negative control (SNC) experiments.

Results Cress length and entropy revealed a time-modulated potency treatment effect, in the absence of a significant main treatment effect. This indicated that the effect of the potency treatment varied significantly across the different experimental days. LCFD yielded a highly significant potency treatment effect. In addition, a significant interaction of treatment with experimental day seems to indicate a modulation of this effect. No significant effects were observed in any of the evaluations of the SNC experiments, indicative of a stable experimental setup and a reliable and specific treatment effect. Neither significant nor strong correlations were found between the four parameters, indicating that they reflect different effects of *Stannum metallicum* 30x on the organism treated.

Keywords
- $CuCl_2$ crystallization
- fractal dimension
- bio-assay
- systematic negative control experiments
- potentized substances

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Conclusion This quadruple characterization of the biological effects of *Stannum metallicum* 30x provides an unprecedented opportunity for basic homeopathy research into, among others, the presumed specificity of homeopathic preparations.

Introduction

Homeopathy is a well-known branch of complementary medicine which, despite its popularity, continues to be discussed as a topic of controversy in the contemporary medical and scientific community as long as the mode of action remains unclear.[1] Unambiguous bio-assays that yield consistent empirical evidence for specific effects of homeopathic remedies are paramount to investigate the mode of action and nature of homeopathic preparations. However, the development of such bio-assays still poses a challenge in homeopathic basic research.[2] Multiple factors seemingly contribute to the difficulties in creating such test systems, including inadequate outcome parameters, underpowered replication trials, and inherent characteristics of homeopathic preparations.[3,4]

Previously, we developed a test system which yielded highly significant evidence for the specific effects of a *Stannum metallicum* 30x preparation in a multi-center replication trial.[5,6] Internal replications, implemented in the pilot experiments[5] and systematic negative control (SNC) experiments[7] conducted in the reproduction trial,[6] indicated a stable experimental setup and a reliable and specific treatment effect. This test system is based on cress (*Lepidium sativum* L.) seed germination and growth for 4 days *in vitro* in either *Stannum metallicum* 30x or control solutions, crushing and aqueous extraction of the cress seedlings and subsequent $CuCl_2$ crystallization of the cress extract and digital textural image analysis of the resulting crystallization patterns.

The method used relies on the emergence of dendritic $CuCl_2$-crystallization patterns when a thin-layer aqueous dihydrate cupric chloride solution ($CuCl_2{\cdot}2H_2O$) is crystallized on a glass plate in the presence of a water-soluble additive (the sample).[8] Additives can be single molecules as well as complex organic matrices.[9–11] The crystallization patterns are additive specific[12] and emerge through a self-organization process of the $CuCl_2$ crystal needles.[13] The branching conditions of the dendrites are influenced by the properties of the additive.[14] The characteristics of the crystallization patterns are evaluated by human visual evaluation using defined criteria,[15–18] and/or by computer-based image analysis.[19–21] $CuCl_2$ crystallization has been applied to a broad range of additives, addressing different research questions ranging from the diagnosis of clinical pathologies in human blood[22,23] to pharmaceutical research,[5,6] but predominantly in (organic) food quality analysis.[18,24,25] The method is used from an ontological holistic stance, which implies evaluating a sample as a whole, thereby complementing analytic data.[26]

In the current experimental setup, the primary outcome parameter quantifies distinct textural features of the crystallization patterns.[19,27] The goal of the present study was to explore whether three additional outcome parameters could further corroborate and characterize the specific effects of *Stannum metallicum* 30x in this bio-assay: (1) the length of the cress seedlings (assessed before the extraction), (2) a second parameter for texture analysis—entropy—to assess additional textural features of the crystallization patterns, and (3) the fractal dimension of the crystallization patterns, as a measure for complexity.

Materials and Methods

General Experimental Procedure

The general experimental procedure (see ►**Fig. 1**) was according to Doesburg et al.[6] *Lepidium sativum* seeds were germinated and grown for 96 hours in suspended plastic zip-lock bags on chromatographic paper wetted with either aqueous homeopathic or control solutions. The seedlings were photographed and subsequently extracted in the corresponding solutions. This extract was mixed with a $CuCl_2$ dihydrate solution and pipetted into Petri dishes. The solution was allowed to evaporate under standardized conditions, resulting in the formation of two-dimensional dendritic $CuCl_2$ crystal patterns. Computer image analysis was used to evaluate the crystallization patterns. Two independent laboratories performed the experiments in parallel (BRAD = Biodynamic Research Association Denmark and CL = Crystal Lab) in Denmark and the Netherlands respectively. The experiments were conducted in a randomized and blinded fashion, and the code was not revealed until the analysis was complete.

Experimental Design

Ten independent verum experiments were performed in parallel in two independent laboratories (BRAD, CL; $n = 10 + 10$ sub-experiments). The verum experiments consisted of 10 independent sets of *Stannum metallicum* 30x and lactose 30x preparations. Experiments ran between December 2012 and June 2013. In each experiment, the two preparations were examined untreated, cell-phone treated and after autoclaving (data analysis of cell-phone and autoclaving treatments will be reported elsewhere). Additionally, 10 independent SNC experiments were performed at both laboratories ($n = 10 + 10$ sub-experiments), in which 6 preparations of distilled H_20, 1x each, produced as 10 independent sample sets, were assessed. The analysis of the data can thus be performed either at the level of experimental number ($n = 10$, allowing the inclusion of the factor 'laboratory' in the analysis of variance [ANOVA] F-tests) or at the level of experimental day ($n = 20$). In each laboratory, the 20 experiments were blinded (coded) and randomized on two levels:

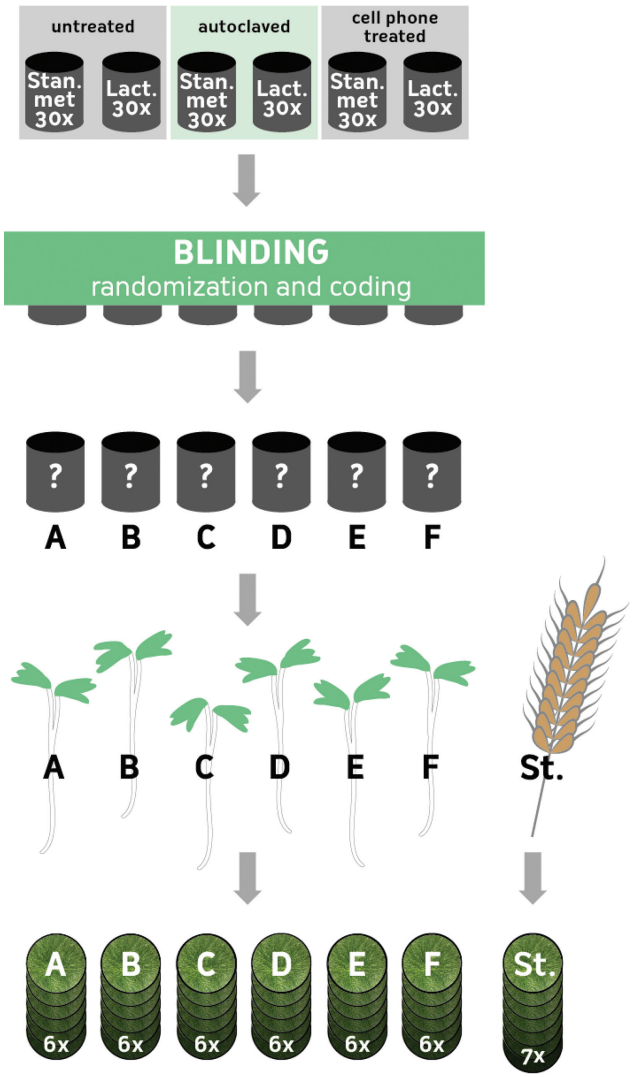

Fig. 1 General design of a verum experiment (reprinted from reference 6, with permission). (1) Homeopathic potencies of *Stannum metallicum* 30x and lactose 30x were prepared and were left untreated or subjected to autoclaving or cell-phone radiation. (2) The six homeopathic potencies were randomized and coded A-F. (3) The preparations were used to cultivate the cress seedlings (wetting 10 filter papers in plastic bags for each of the six experimental conditions). (4) An aqueous extract was prepared from crushed seedlings after a 96h growth period, extracted in the corresponding potencies. (5) For each experimental condition, six crystallization plates were prepared. In addition, seven crystallization plates were prepared using an open (non-coded) internal standard (freeze-dried wheat).

with respect to the experimental series (verum or SNC) and with respect to the individual treatments within the experiments. Six cress extracts, corresponding to the six experimental parameters or the six SNC preparations, were mixed with a $CuCl_2$ dihydrate solution and allowed to crystallize under defined conditions (29°C; initial rH 49%, air velocity 4 cm s^{-1}; HygroClip S, Rotronic AG, Switzerland) in specially designed climatic chambers.[28,29] Crystallization was performed in six-fold repetition, resulting in 36 crystallization plates per experimental day.

The experimental design, which included 10 SNC experiments in addition to the 10 verum experiments per laboratory, allowed control of the reproducibility and experimental stability of the bio-assay throughout the study. Reproducibility could be examined between laboratories and between experiments by comparison of the results as a function of individual experiments. For more detailed additional information, see the Materials and Methods section of Doesburg et al.[6]

Seed Germination and Growth

The applied germination procedure was a modification of that developed by Baumgartner & Flückiger.[30] Germination took place on filter papers (blotting paper 151B; 85 × 140 mm; 87 g/m^2; thickness 0.17 mm; Frisenette, Knebel, Denmark), placed in plastic bags (MiNiGRiP Colorline; type 11–16; 100 × 150 × 0.05 mm; JOKA Plastic-Emballage A/S, Holte, Denmark). Germination was performed on the "smooth" side: that is, the side free of loose fibers. A volume of 3.00 mL of a given preparation was pipetted onto each filter paper. A total of 10 filter papers were used for each preparation. When the solution was absorbed evenly over the filter paper, 16 seeds were placed on the paper. To minimize the effect of the order of processing, a 12 minutes time block was applied to the start of germination of each preparation. Minimal air access into the bags was achieved by manually expanding the upper closing area of the bags on both sides. The bags were placed in a germination box (polyethylene; 200 × 315 × 450 mm) and hung vertically from two stainless steel rods (diameter 3 mm) with no contact to the bottom and lid. The bags were positioned on the rods according to the order of processing of the six preparations. Bags belonging to the different preparations were separated by means of a plastic bag that contained a filter paper that was covered with two layers of aluminum foil. Similarly, a piece of cardboard covered with a double layer of aluminum foil was placed between the two rows of plastic bags. The germination box was covered with a plastic lid and placed in a heating cabinet at 18°C (± 1°C) on a perforated plate. After 2.5 hours, the seeds had developed a mucous sphere, allowing the 16 seeds per bag to be aligned 9 cm from the bottom of the filter paper at a distance of 2 to 3 mm from each other, followed by another 93.5 hours of incubation. Seedlings typically reached a total shoot and root length of approximately 9 cm after this period.

Preparation of Seedlings

After the 96 hours growth period, a total of five plastic zipper bags containing the seedlings were cut open on both longitudinal sides, approximately 3 mm from the filter paper and also approximately 3 mm above the paper. The front of the bag was opened, and the top of the paper was bent backward, making the seedlings accessible for individual manual selection. Seedlings with a minimum root length of 6.5 cm were selected from each bag, which generally resulted in 8 to 12 seedlings per bag. Seedlings with abnormal shape, color, or signs of fungal growth were discarded. The brown seed coats, usually separated from the seedlings after germination, were discarded. The seedlings were collected in a Petri dish that was placed on a balance. For each treatment, 1.50 g of seedlings was collected from the first five corresponding bags, on

average 10 seedlings per bag, each seedling weighing approximately 0.03 g. The seedlings were put in a mortar (porcelain; 160 mL; Ø = 90 mm; Haldenwanger, Waldkraiburg, Germany), containing 10.0 mL of a given preparation. The mortar was covered with Parafilm M (Pechiney, Menasha, USA) until seedlings from all treatments had been prepared. This procedure was repeated with the remaining five plastic bags per treatment until each mortar contained a total of 3.00 g seedlings. This double step of seedling collection was used in order to further minimize a potential processing order effect. The seedlings were crushed with a pestle, making diagonal movements for 2 minutes so that no intact leaf or root parts were observed in the solution. This was followed by 1 minute of lemniscate motion, which further homogenized the leaf and root parts. Pestle and mortar were each flushed with 8.50 mL from the appropriate preparation, to produce a 10% solution on a weight basis (3.00 g seedlings; 27.00 mL potency; in total 30.00 g solution). Extraction of the seedlings in the preparation applied during germination was performed to obtain a maximal differentiation between experimental groups.

The solutions were transferred to wide-necked 100 mL Erlenmeyer flasks. Each flask was covered with Parafilm and left until all treatments were completed. The solutions were extracted on a horizontal shaker (Heidolph Unimax 2010) at 125 rpm for 45 minutes. The extracts were filtered for 3 minutes over a nylon filter (pore size 150 µm; 03–150/38; Sefar AG, Heiden, Switzerland), placed in a glass funnel (upper diameter 70 mm, tube length 70 mm, tube diameter 10 mm), and placed in pre-weighed wide-necked 100 mL Erlenmeyer flasks. The solutions used for $CuCl_2$ crystallization analysis were prepared on the basis of an extract of 300 mg substance (seedlings) per crystallization plate, combined with 150 mg of dihydrate $CuCl_2$ (copper (II) chloride dihydrate; Merck, article no. 1.02733.1000; pro analysis) from a 5% stock solution, generating a volume of 6.0 mL per plate. This yielded six crystallization plates (replicates) per *Lepidium sativum* extract preparation per experiment, resulting in a total of 36 crystallization plates for the 6 *Lepidium sativum* extract preparations.

Cress Length Measurements

After the 96 hours germination period, the seedlings on the chromatography papers were photographed in their plastic zipper bags. The contrast between seedlings and chromatographic paper was enhanced by lateral LED illumination and the use of a black background. To calibrate the seedling lengths, the zipper bags were photographed alongside millimeter paper. True curve length of the plants was measured by means of the software "Tracking 0.2.6" (A. Fritschy, Informatik-Lösungen, Zürich, Switzerland) using a Summagraphics SummaSketch III digitizing tablet (GTCO CalComp, Columbia, United States) connected to an Apple iBook G3 computer (Apple, Cupertino, United States).

Scanning and Computerized Texture Analysis

Scanning of the crystallization plates, gray level co-occurrence matrix (GLCM) transformation and selecting of the circular regions of interest (ROIs) for texture analysis (TA) purposes was described previously in Doesburg et al.[6] In short, the crystallization plates were scanned as 8-bit RGB images with a resolution of 600dpi using a Umax PowerLook III scanner. TA was performed on all scanned crystallization plates of the 20 CL and 20 BRAD experiments, besides those discarded due to technical errors (see below). TA is based on GLCM evaluation, thereby taking the spatial relationship of pixels in each image into account[27] From the GLCM, numerical features are computed that can be used for a more compact representation of the texture.[31] For instance, TA variable 'cluster_shade' is defined as a measure of skewness (asymmetry) and uniformity. A higher cluster shade output implies a greater degree of asymmetry, whereas TA variable 'entropy' is a measure of information content, measuring the randomness of intensity distribution. A more complex image will have a higher entropy than a non-complex image.

Local Connected Fractal Dimension

The local connected fractal dimension (LCFD) of the crystallization patterns was measured by means of the Fraclac plugin[32] for Image J (version 1.51i).[33] Prior to fractal analysis, the 8-bit RGB crystallization scans were cropped from $2{,}112 \times 2{,}112$ pixels to $2{,}080 \times 2{,}080$ (98.5% of the total radius), to remove the fringe, where crystal growth is undefined due to the presence of the perspex rim. The scans were binarized by means of standard ImageJ binarization, and thresholding was based on the histogram of the entire image.

The fractal analysis estimates an object's self-similarity qualities: that is, the degree to which a part of it appears to be whole at various scales.[34] LCFD provides a distribution of local variation in complexity, as opposed to the global fractal dimension. Hereto, LCFD calculates the total number of pixels connected to a seed pixel in a series of increasing oval calibers centered around the seed pixel. The applied oval calibers (7 in total) were determined empirically and ranged from 1 to 13 pixels in diameter. Connected pixels are all foreground pixels that are within the 8×8 local environment of the seed pixel. This routine is repeatedly applied to all connected pixels within each caliber.[32] This process is iterated over every 5th pixel horizontally and vertically for the entire crystallization pattern (see ►**Fig. 2** for a color-coded crystallization pattern according to the LCFD).

Missing Data

Fourteen crystallization plates (out of a total of $40 \times 36 = 1{,}440$) were discarded due to technical failures, mainly caused by leaking solution under the surrounding acrylic ring. Of these 14 plates, 6 originated from verum and 8 from SNC experiments. Thus, in total, 1,426 crystallization plates were used for computerized TA and LCFD analysis.

Skewness Correction

Skewed data were normalized to $-0.5 < \text{skewness} < 0.5$ by applying increasing cut-offs of the data, in an iterative process. This approach maintains the units of the variables, as opposed to square root or logarithmic data transformation procedures.

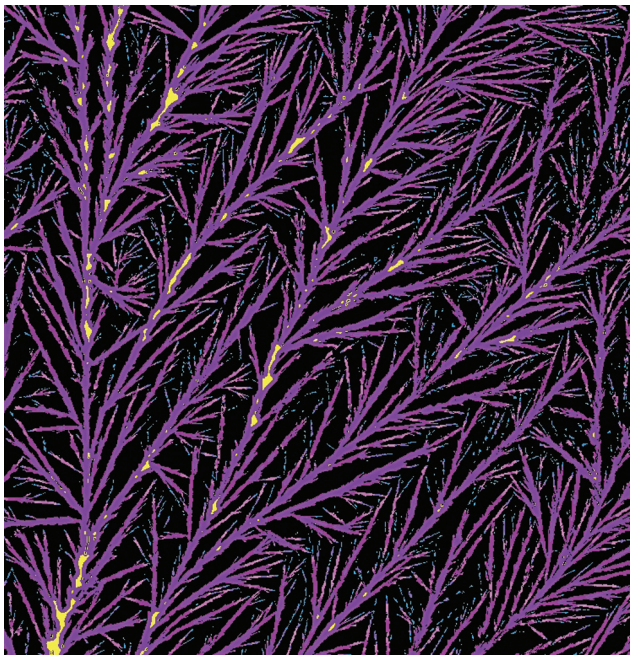

Fig. 2 Color coded cress crystallization pattern according to the fractal dimension (FD) at each local 1 × 1 pixel area. To enhance visual perception, coloring is presented at a 1 × 1 pixel area, instead of the applied 5 × 5 pixel area. Color scaling: FD 0.2–0.6 ; FD 0.7–1.0 ; FD 1.3–1.5 ; FD 1.5–1.7 ; FD 1.7–1.9 ; FD 1.9–2.0 .

Statistical Analysis

ANOVA F-tests were performed with software Statistica 6.0 for Windows (StatSoft Inc., Tulsa, USA). Graphs were plotted with Rstudio Version 1.3.1093[35] and the packages "ggplot2" version 3.2.1[36] and "ggpubr" version 0.6.0.

Results

Cress Length Analysis

Cress length data were considerably skewed (−1.46). Skewness correction was performed with cut-off values < 70 and > 141 mm, resulting in a skewness decrease from −1.46 to −0.36, and 15.73% data reduction. All further analyses were performed with the skewness corrected data.

Cress length data were analyzed by a three-way ANOVA, using (1) laboratory (BRAD, CL), (2) experimental number ($n = 10$) and (3) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$) as the three independent variables. Results are presented in ►**Table 1a**.

For the verum and SNC experiments, highly significant differences in absolute values were found for the experimental number and the laboratories, indicating that the seedling lengths differed over the experimental days and between both laboratories; seedlings at BRAD were on average 4.1% shorter compared to seedlings at CL, which may be due to a slightly higher temperature in the CL heating cabinet. Furthermore, a highly significant interaction was found between laboratory and experimental number ($p < 0.0001$, ►**Table 1a**, interaction 1–2) for the verum and SNC experiments, which is due to the variation in absolute values of the cress length between the laboratories and the individual experiments.

No significant overall treatment effect of *Stannum met.* 30x was found, albeit a significant interaction was observed for the verum experiments between potency treatment and laboratory ($p = 0.0297$, ►**Table 1a**, interaction 1–3). Fisher-LSD post-hoc analysis indicated a significant treatment effect for CL only ($p = 0.0145$). Additionally, a highly significant interaction was found between potency treatment, laboratory and experimental number ($p = 0.0043$, ►**Table 1a**, interaction 1–2–3) for the verum experiments only, indicating that the effect of the homeopathic potencies was modulated by a factor associated with the experimental number and/or the laboratories.

To investigate whether this three-way interaction was caused by time-dependent effects of *Stannum metallicum* 30x, we conducted a two-way ANOVA, using (1) experimental day ($n = 20$) and (2) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$) as the two independent variables. Results are presented in ►**Table 1b**. We found a highly significant interaction between potency treatment and experimental day ($p = 0.0014$, ►**Table 1b**, interaction 1–2) for the verum experiments only. When the percentage difference between *Stannum metallicum* 30x and lactose 30x treated cress was plotted over the experimental days (see ►**Fig. 3**) and compared with the SNC experiments, an increased variability of the cress length for the verum data became apparent. Especially on experimental days 8, 11, 15 and 17, *Stannum metallicum* 30x seemed to reduce plant growth, while it seemed to enhance plant growth on day 5 and 13.

Table 1a Results from three-way ANOVA F-tests for the cress length (main effects and interaction)

Parameter	Main effects			Effect interactions			
	(1) Lab	(2) Exp. Num.	(3) Treatment	1–2	1–3	2–3	1–2–3
Verum experiments (*p*-value)							
Cress length	**<0.0001**	**<0.0001**	0.2811	**<0.0001**	**0.0297**	0.1378	**0.0043**
SNC experiments (*p*-value)							
Cress length	**<0.0001**	**0.0091**	0.8034	**<0.0001**	0.1519	0.6907	0.8621

Abbreviations: ANOVA, analysis of variance; Exp. Num., experimental number; Lab, laboratory; SNC, systematic negative control.
Note: Independent experimental parameters were (1) Lab (BRAD, CL; $n = 2$), (2) Exp. Num. (1–10; $n = 10$) and (3) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$). Each experimental parameter combination (statistical treatment cell) was assessed by approximately 160 seedlings. Significant effects below $p < 0.05$ are shown in bold red font.

Table 1b Results from two-way ANOVA F-tests for the cress length (main effects and interaction)

Parameter	Main effects		Effect interactions
	(1) Exp. Day	(2) Treatment	1–2
Verum experiments (*p*-value)			
Cress length	**<0.0001**	0.2811	**0.0014**
SNC experiments (*p*-value)			
Cress length	**<0.0001**	0.8034	0.8256

Abbreviations: ANOVA, analysis of variance; Exp. Day., experimental day; SNC, systematic negative control.
Note: Independent experimental parameters were (1) Exp. Day (1–20; $n = 20$) and (2) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$). Each experimental parameter combination (statistical treatment cell) was assessed by approximately 160 seedlings. Significant effects below $p < 0.05$ are shown in bold red font.

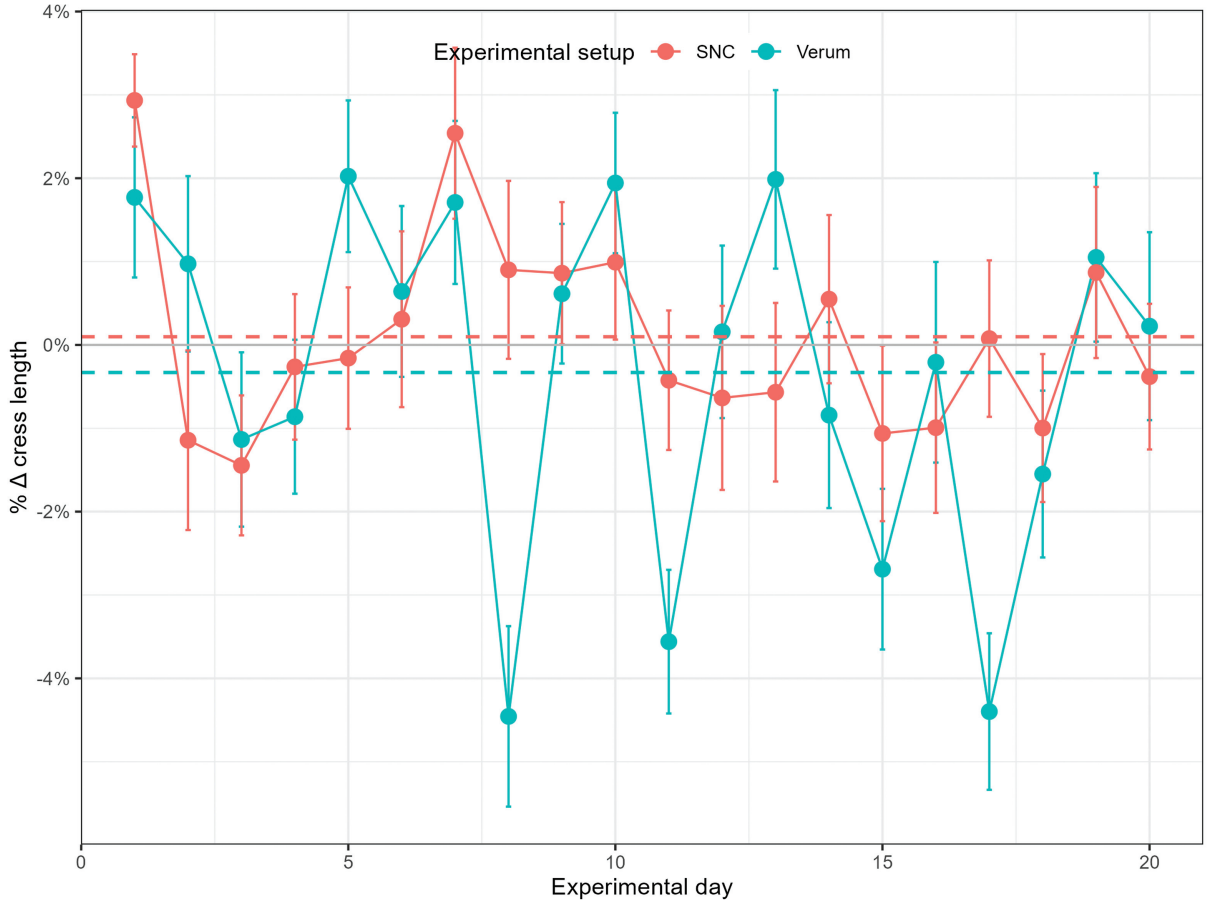

Fig. 3 Percentage difference $(1 - \frac{cresslengthStannummet.30x}{cresslengthLactose30x})$ for the verum experiments (blue dots, mean ± SE), and the systematic negative control (SNC) experiments (red dots, mean ± SE). The overall grand mean values over the 20 experimental days for the verum and SNC experiments are indicated by the similarly colored dashed lines (mean verum difference = −0.33%; SNC = 0.10%).

Texture Analysis Variable, Entropy

The degree of skewness of the entropy combining verum and SNC data was within the threshold of $-0.5 <$ skewness < 0.5 (0.03); therefore the raw data were used for all further analyses.

Consistent with the cress length analysis, TA data were analyzed by a three-way ANOVA, using (1) laboratory (BRAD, CL), (2) experimental number ($n = 10$) and (3) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$) as the three independent variables. Calculations were based on the entire crystallization plate diameter (ROI, 0–100%). Results are presented in ►**Table 2a**. The results of the TA variable cluster_shade are added for reference purposes.

Similar to the cluster_shade data, TA variable entropy yielded highly significant differences in absolute values between the two laboratories for the verum and SNC experiments. For the verum experiments only, significant differences in absolute values were found between the experimental numbers ($p = 0.0152$, ►**Table 2a**, main effect 2).

Table 2a Results from three-way ANOVA F-tests for the TA variables cluster_shade and entropy (main effects and interaction)

Parameter	Main effects			Effect interactions			
	(1) Lab	(2) Exp. Num.	(3) Treatment	1–2	1–3	2–3	1–2–3
Verum experiments (*p*-value)							
Cluster_shade	**<0.0001**	**0.0152**	**0.0336**	**<0.0001**	0.1363	**0.0409**	0.3801
Entropy	**<0.0001**	**0.0152**	0.8826	**0.0340**	0.4248	0.5980	**0.0031**
SNC experiments (*p*-value)							
Cluster_shade	**<0.0001**	**0.0011**	0.2397	**<0.0001**	0.8439	0.3575	0.0682
Entropy	**<0.0001**	0.1599	0.3321	**<0.0001**	0.3298	0.3648	0.2298

Abbreviations: ANOVA, analysis of variance; Exp. Num., experimental number; Lab, laboratory; TA, texture analysis; SNC, systematic negative control.
Note: Independent experimental parameters were (1) Lab (BRAD, CL; $n = 2$), (2) Exp. Num. (1–10; $n = 10$) and (3) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$). Each experimental parameter combination (statistical treatment cell) was assessed by six crystallization patterns. Significant effects below $p < 0.05$ are shown in bold red font. Cluster_shade data from[6] are added for reference purposes.

Contrary to cluster_shade, however, no significant main treatment effect was found for entropy. Yet, a highly significant interaction was found between potency treatment, laboratory and experimental number ($p = 0.0031$, ►**Table 2a**, interaction 1–2–3) for the verum experiments only. This indicates that the effect of *Stannum metallicum* 30x was modulated by a factor associated with the experimental number and/or the laboratories. Similarly to the analysis of the cress seedling length, we assumed this threefold interaction to be caused by time-varying effects of *Stannum metallicum* 30x. To confirm this assumption, we conducted a two-way ANOVA, using (1) experimental day ($n = 20$) and (2) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$) as the two independent variables. Results are presented in ►**Table 2b**. In line with our assumption, we found a significant interaction between potency treatment and experimental day ($p = 0.0242$, ►**Table 2b**, interaction 1–2) for the verum experiments only.

When the percentage difference between *Stannum metallicum* 30x and the lactose 30x for the verum and SNC experiments was plotted over the experimental days (see ►**Fig. 4**), an increased variability of entropy for the verum data became apparent. The entropy values seemed to be lower for the *Stannum metallicum* 30x treatment for experiments 4, 5 and 17 and higher for experiments 3 and 15.

Local Connected Fractal Dimension Analysis

LCFD data were considerably skewed (0.95). Skewness correction was performed with cut-off values < 1.52 and > 1.61, resulting in a skewness decrease from 0.95 to 0.32 and 4.42% data reduction. All further analyses were performed with the skewness-corrected data.

LCFD data were analyzed by a three-way ANOVA, using (1) laboratory (BRAD, CL), (2) experimental number ($n = 10$) and (3) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$) as the three independent variables. Results are presented in ►**Table 3a**.

For the verum and SNC experiments, highly significant differences in absolute values were found between the experimental numbers. An interaction was observed between laboratory and experimental numbers for the verum and SNC experiments ($p = 0.0003$ and 0.0386, respectively, ►**Table 3a**, interaction 1–2), indicating that the absolute LCFD values varied between the two laboratories over the experimental numbers.

Table 2b Results from two-way ANOVA F-tests for the TA variables cluster_shade and entropy (main effects and interaction)

Parameter	Main effects		Effect interactions
	(1) Exp. Day	(2) Treatment	1–2
Verum experiments (*p*-value)			
Cluster_shade	**<0.0001**	**0.0336**	0.0647
Entropy	**<0.0001**	0.8826	**0.0242**
SNC experiments (*p*-value)			
Cluster_shade	**<0.0001**	0.2397	0.1401
Entropy	**<0.0001**	0.3321	0.2582

Abbreviations: ANOVA, analysis of variance; Exp. Day, experimental day; TA, texture analysis; SNC, systematic negative control.
Note: Independent experimental parameters were (1) Exp. Day (1–20; $n = 20$) and (2) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$). Each experimental parameter combination (statistical treatment cell) was assessed by six crystallization patterns. Significant effects below $p < 0.05$ are shown in bold red font. Cluster_shade data from[6] are added for reference purposes.

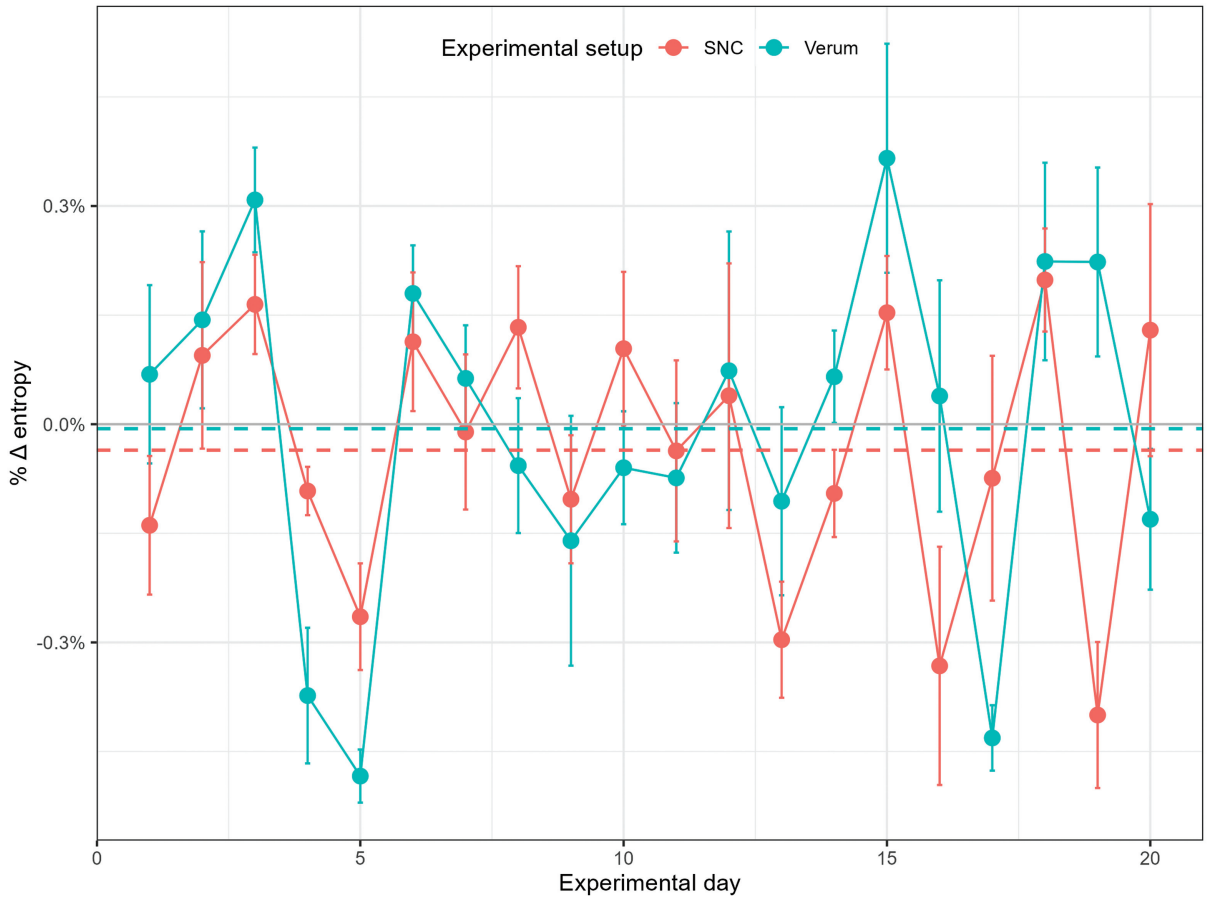

Fig. 4 Percentage difference $(1 - \frac{EntropyStannummet.30x}{EntropyLactose30x})$ for the verum experiments (blue dots, mean ± SE), and the systematic negative control (SNC) experiments (red dots, mean ± SE). The overall mean values over the 20 experimental days for the verum and SNC experiments are indicated by the similarly colored dashed lines (mean verum difference = −0.006%; SNC = −0.030%).

Regarding the treatment with *Stannum metallicum* 30x, a highly significant main effect was found for the verum experiments only, meaning that a difference was found in the local fractal dimension of crystallization patterns of cress seedlings germinated in *Stannum metallicum* 30x versus lactose 30x. Higher mean LCFD values were found for *Stannum metallicum* 30x compared to lactose 30x (1.572 vs. 1.567, respectively), indicative of a higher mean local complexity of the crystallization patterns derived from the cress grown in *Stannum metallicum* 30x. For the spatial region of the crystal patterns where the effect of the potency treatment is most evident in the LCFD, the reader is referred to ►**Supplementary File 1** (available in the online version).

A borderline significant interaction was found between experimental number and potency treatment for the verum experiments only, indicating a slight variability in the potency treatment effect as a function of experimental number ($p = 0.0474$, ►**Table 3a**, interaction 2–3). This time-modulated effect of *Stannum metallicum* 30x could be confirmed by means of a two-way ANOVA, using (1) experimental day ($n = 20$) and

Table 3a Results from three-way ANOVA F-tests for the LCFD (main effects and interaction)

Parameter	Main effects			Effect interactions			
	(1) Lab	(2) Exp. Num.	(3) Treatment	1–2	1–3	2–3	1–2–3
Verum experiments (*p*-value)							
LCFD	0.2065	**<0.0001**	**0.0083**	**0.0003**	0.2254	**0.0474**	0.0671
SNC experiments (*p*-value)							
LCFD	0.5056	**0.0091**	0.7137	**0.0386**	0.2488	0.2472	0.1999

Abbreviations: ANOVA, analysis of variance; Exp. Num, experimental number; Lab, laboratory; LCFD, local connected fractal dimension; SNC, systematic negative control.
Note: Independent experimental parameters were (1) Lab (BRAD, CL; $n = 2$), (2) Exp. Num. (1–10; $n = 10$) and (3) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$). Each experimental parameter combination (statistical treatment cell) was assessed by six crystallization patterns. Significant effects below $p < 0.05$ are shown in bold red font.

Table 3b Results from two-way ANOVA F-tests for the LCFD (main effects and interaction)

Parameter	Main effects		Effect interactions
	(1) Exp. day	(2) Treatment	1–2
Verum experiments (*p*-value)			
LCFD	**<0.0001**	**0.0083**	**0.0214**
SNC experiments (*p*-value)			
LCFD	**0.0050**	0.7137	0.2096

Abbreviations: ANOVA, analysis of variance; Exp. Day, experimental day; LCFD, local connected fractal dimension; SNC, systematic negative control.
Note: Independent experimental parameters were (1) Exp. Day (1–20; $n = 20$) and (2) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$). Each experimental parameter combination (statistical treatment cell) was assessed by six crystallization patterns. Significant effects below $p < 0.05$ are shown in bold red font.

(2) treatment (*Stannum met.* 30x or lactose 30x [verum experiments] or water 1x and water 1x [SNC experiments]; $n = 2$) as the two independent variables. Results are presented in ►**Table 3b**. Both the verum and SNC experiments showed a highly significant experimental day effect for the absolute LCFD values. In contrast, only for the verum experiments highly significant differences between the potency treatments were found and a significant interaction between experimental day and treatment ($p = 0.0083$ and 0.0214, respectively, ►**Table 3b**, main effect 2 and interaction 1–2).

The absence of both significant differences between the SNC water control samples and interactions between water control treatment and experimental number indicate a stable experimental setup and a reliable treatment effect of *Stannum metallicum* 30x occurring in the verum experiments. A graphical representation of the dependency of the LCFD on the treatment is given in ►**Fig. 5** for the verum and SNC experiments.

Correlations Between the Parameters

To determine whether cress length, the TA variable entropy and LCFD potentially provide supplementary information regarding specific effects of the *Stannum metallicum* 30x treatment, correlations between cress length, entropy, the previously used TA variable cluster_shade and LCFD were carried out (see ►**Fig. 6**). Neither significant nor strong correlations were found between the four parameters, indicating that cress length, entropy and LCFD are indeed independent novel outcome parameters for this test system.

Processing Order Effect

Extensive measures were taken to minimize unwanted effects in the processing of the six treatments. Notwithstanding the measures taken, we tested the possibility of a laboratory processing order effect for the four independent parameters. The reader is referred to ►**Supplementary File 1** (available in the online version) for details.

Discussion

Three Additional Outcome Parameters

Previously, we had developed a test system which yielded highly significant evidence for specific effects of a *Stannum metallicum* 30x preparation in a multi-center replication trial.[5,6] This test system is based on cress seed germination and growth in homeopathic or control samples, $CuCl_2$

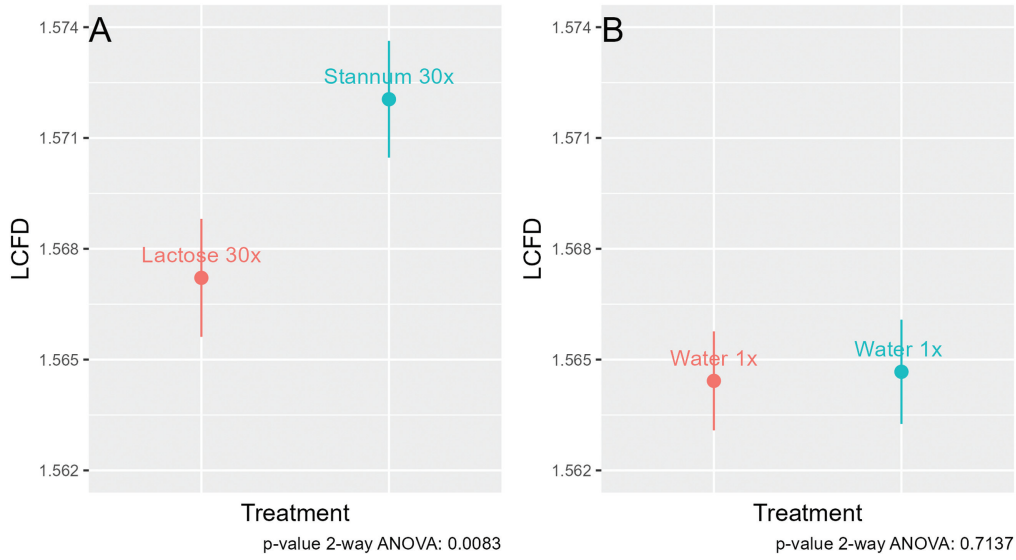

Fig. 5 LCFD (mean ± SE) of the cress seedling crystallization patterns for the verum (plot A, left) and the SNC experiments (plot B, right).

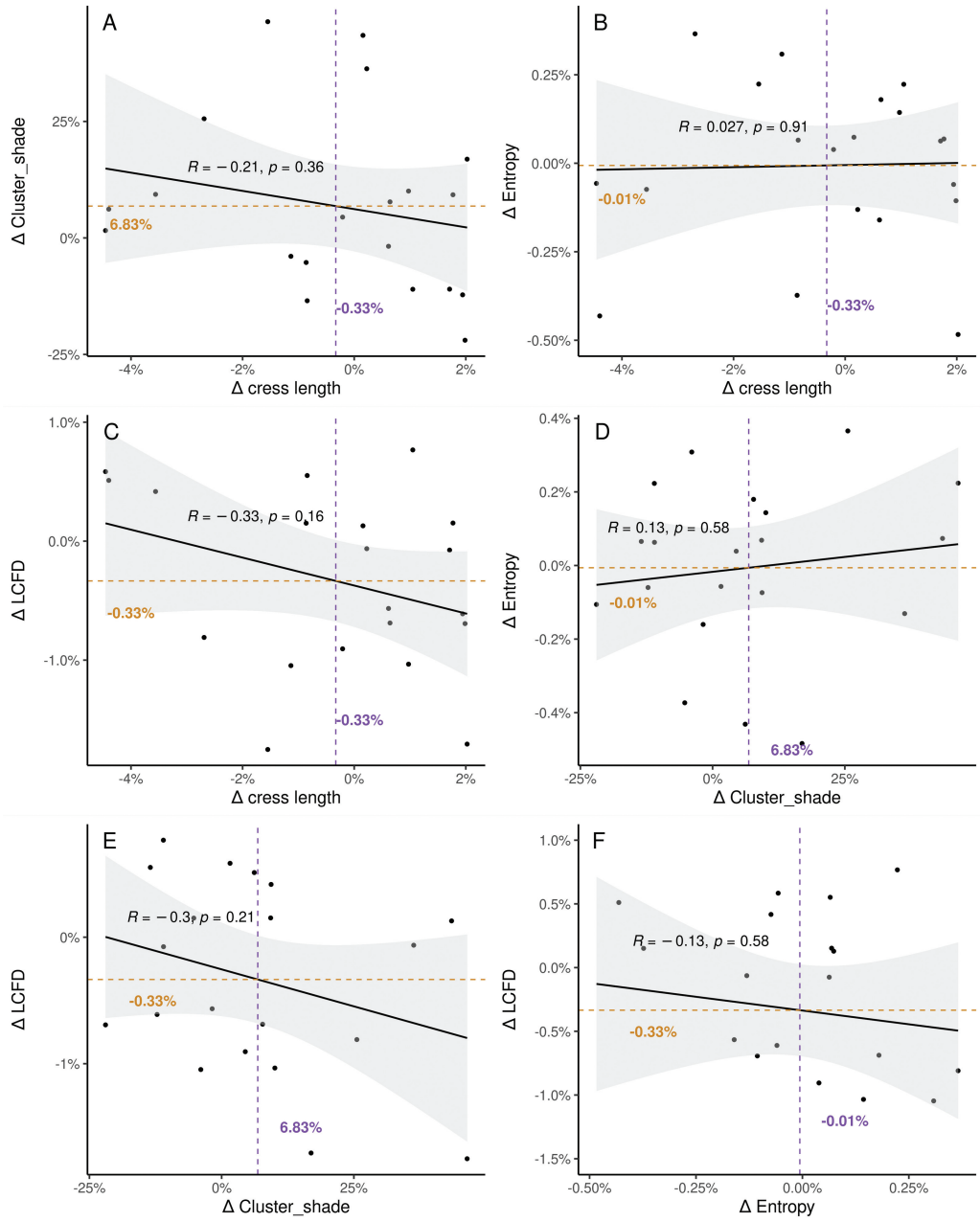

Fig. 6 Relative percentage treatment effect $\%\frac{Var.Lactose30x-Var.Stannummet.30x}{Var.Lactose30x}$ for the 20 individual experimental days for: (**A**) cress length (x-axis) versus cluster_shade (y-axis), (**B**) cress length (x-axis) versus entropy (y-axis), (**C**) cress length (x-axis) versus LCFD (y-axis), (**D**) cluster_shade (x-axis) versus entropy (y-axis), (**E**) cluster_shade (x-axis) versus LCFD (y-axis), (**F**) entropy (x-axis) versus LCFD (y-axis). In black the regression line, with correlation coefficient R, significance level *p* and 95% confidence interval (*gray area*). Red dashed line depicts the mean y-axis variable's treatment effect; blue dashed line depicts the mean x-axis variable's treatment effect.

crystallization of the cress extract, and subsequent digital TA of the resulting crystallization patterns. The goal of the present study was to explore whether three novel outcome parameters could further corroborate and possibly characterize the specific effects of *Stannum metallicum* 30x. To this end, (1) cress seedling length, (2) a second TA parameter, entropy, and (3) the LCFD of crystallization patterns as a measure of complexity were considered.

Cress Length Analysis

Cress length analysis revealed a time-modulated potency treatment effect (two-way ANOVA, p = 0.0014, ►**Table 1b**, interaction 1–2), in the absence of a significant main treatment effect. This indicated that the effect of the potency treatment varied significantly across the different experimental days. Visually, this effect could be made apparent by plotting the percentage difference between the *Stannum metallicum* 30x and the lactose 30x treated cress for the verum and SNC experiments over the experimental days (see ►**Fig. 3**). Especially on experimental days 8, 11, 15 and 17, *Stannum metallicum* 30x seemed to reduce plant growth, while it seemed to enhance plant growth on days 5 and 13. This time-modulated effect was absent in the SNC experiments, indicating a stable experimental setup.

Texture Analysis Variable, Entropy

As described in Doesburg et al,[6] many of the 15 TA variables[27] were correlated to each other. Within the verum data, two main groups of variables were identified that were closely correlated within each group, but not or only weakly correlated with those of the other group: group I, cluster_shade and diagonal_moment ($r = -0.97$); group II, all other TA variables with the exception of variable cluster_prominence ($r > 0.71$). Doesburg et al[6] focused on the variables of group I (cluster_shade and diagonal_moment), which had been identified in the Baumgartner et al[5] study as the outcome variables of primary interest. In the present study, we focused on a representative of the group II variables (entropy). The results of the group I variable cluster_shade are added for reference purposes.

Cluster_shade yielded a significant homeopathic treatment effect in the dataset analyzed here ($p = 0.034$).[6] A graphical representation of the mean values of cluster_shade as a function of the treatment is given in ►**Fig. 7** for the verum and SNC experiments. Entropy, on the other hand, yielded a time-modulated potency treatment effect (two-way ANOVA, $p = 0.0242$, ►**Table 2b**, interaction 1–2), in the absence of a significant main treatment effect. Visually, this effect could be made apparent by plotting the percentage difference between *Stannum metallicum* 30x and the lactose 30x for the verum and SNC experiments over the experimental days (see ►**Fig. 4**). The entropy values seemed to be lower for the *Stannum metallicum* 30x treatment for experiments 4, 5 and 17 and higher for experiments 3 and 15. This time-modulated effect was absent in the SNC experiments, indicating a stable experimental setup.

Local Connected Fractal Dimension

LCFD yielded a highly significant potency treatment effect (two-way ANOVA, $p = 0.0083$, ►**Table 3b**, main effect 2, and ►**Fig. 5**), meaning that a mean difference was found in the local fractal dimension of crystallization patterns of cress seedlings germinated in *Stannum metallicum* 30x versus lactose 30x across all verum experiments performed. Results revealed a higher mean local complexity of the crystallization patterns derived from the cress grown in *Stannum metallicum* 30x compared to lactose 30x. In addition, a significant interaction of treatment with experimental day seems to indicate a modulation of this effect. The SNC experiments indicated a stable experimental setup.

Correlations between the Parameters

Neither significant nor strong correlations were found between cress length, cluster_shade and/or entropy and LCFD, indicating that these four outcome parameters are independent and depict different effects of *Stannum metallicum* 30x on the organism treated, or—in other words—they carry independent signals.

Interpretation of Multiple Independent Signals

In this article, we identified three additional outcome parameters for the cress-$CuCl_2$-crystallization system, bringing the total to four independent outcomes. To understand the occurrence of multiple independent signals in the reaction to an intervention, the systemic response of plants to stress presents a useful example. For instance, arsenic treatment of rice seedlings shows a marked decrease in multiple outcome parameters such as germination rate, shoot and root elongation and plant biomass.[37] Likewise, heat stress causes a variety of and often detrimental changes in plant growth, development, physiological processes and yield.[38] Similarly, $CuCl_2$ crystallization of aged or processed plant extracts results in a multi-fold response in the arrangement of the crystallization pattern, such as the degree of branching, length and density of the side needles, the homogeneity of the branching angle[15–18] and the fractal dimension.[39] Other examples include the differential effects of

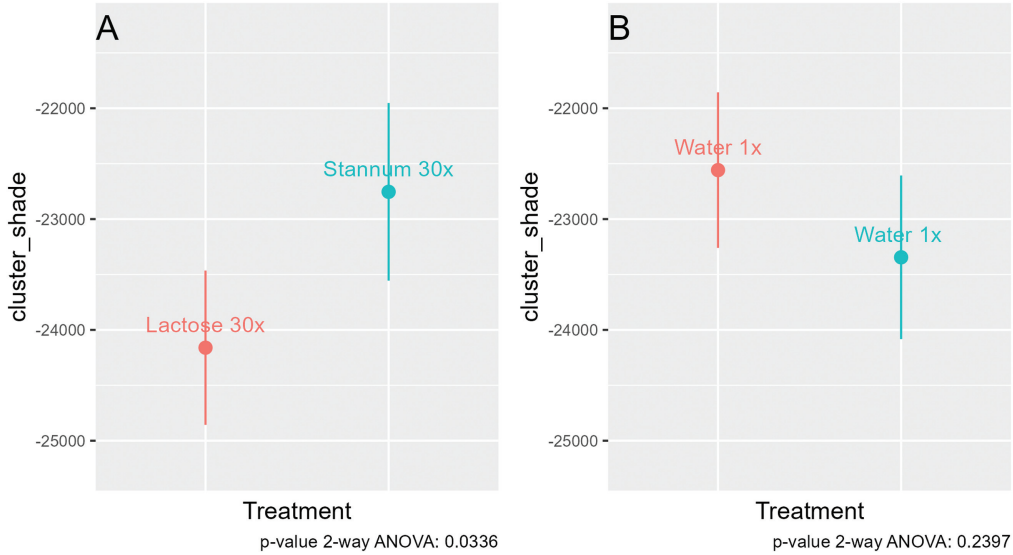

Fig. 7 TA-parameter cluster_shade (mean ± SE) of the cress seedling crystallization patterns for the verum (plot A, left), and the SNC experiments (plot B, right). Original data from reference 6.

homeopathic remedies at different levels of the human being, as identified in remedy provings.[40]

In the case of the present cress-$CuCl_2$-crystallization assay, cress length is an outcome parameter that needs no further explanation. The meaning of the three further outcome parameters of the $CuCl_2$-crystallization assay, originating from texture and fractality of the resulting crystallization patterns (cluster_shade, entropy and LCFD), is less clear, however. They can be generally understood as aspects of a fingerprint metabolomic assay,[6] and their specific significance is not yet determined. Further investigations are necessary to achieve a deepened understanding. Nevertheless, the present statistical analysis suggests a total of four independent outcome parameters representing different reactions of cress to *Stannum metallicum* 30x.

Conclusions and Outlook

Together with the previously identified primary outcome parameter cluster_shade,[5] the three complementary outcome parameters presented in this article (cress length, entropy and LCFD) provide a quadruple characterization opportunity of the biological effects of *Stannum metallicum* 30x on cress seedlings, based on different aspects of the bioassay applied here. This provides an unprecedented opportunity for basic homeopathic research into, among others, the mode of action of homeopathic remedies.

No significant effects were observed in any of the evaluations of the SNC experiments, which yields very strong evidence for the stability of the experimental setup, and which is in favor of the absence of false-positive results in the verum experiments.

Multidimensional outcomes are a mandatory precondition to investigate the presumed specificity of homeopathic preparations. In clinical research, differential effects of *Natrium muriaticum* 30c and *Arsenicum album* 30c were identified against placebo in a randomized and blinded pathogenetic trial.[41] This was possible because the remedy pictures of these two preparations differ from each other on many different levels of the human being. There are still only a few basic research investigations that have observed differing effects of several potentized substances against controls.[42–44] Due to its multidimensionality, the present cress-$CuCl_2$-crystallization assay can be used to further investigate the presumed specificity of homeopathic preparations.

Highlights

- Previously we developed a test system which yielded highly significant evidence for specific effects of a *Stannum metallicum* 30x preparation in a multi-center replication trial.
- In this study, three new outcome parameters were identified, which exhibited neither significant nor strong correlations, indicating that they reflect different effects of *Stannum metallicum* 30x on the organism treated.
- This quadruple characterization of the biological effects of *Stannum metallicum* 30x provides an unprecedented opportunity for basic homeopathy research into, among others, the presumed specificity of homeopathic preparations.

Supplementary Material

Supplementary File 1: Supplementary results.

Conflict of Interest

None declared.

Acknowledgements

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