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Autoclavation of Aqueous *Stannum metallicum* 30x Modifies Its Effects in a Cress Seedling–$CuCl_2$ Crystallisation Bioassay

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Abstract

Background/Objectives: The widespread use of autoclavation as the preferred terminal sterilisation method for sterile homeopathic preparations, including ophthalmic and parenteral dosage forms, raises the question of whether this standard manufacturing step affects their biological activity. For ultramolecular homeopathic preparations, this question cannot be addressed by conventional physicochemical stability testing, since no original substance remains in measurable quantities. We investigated whether autoclavation of *Stannum metallicum* 30x affected its response in a previously validated *Lepidium sativum* $CuCl_2$ crystallisation bioassay with four independent outcome parameters. **Methods:** The present autoclavation study was conducted within the same experimental series as a parallel microwave irradiation study, providing a well-controlled basis for comparing the effects of two distinct physical treatments on the same preparation. Experiments were performed in parallel at two independent laboratories, and experimental stability was monitored throughout with systematic negative control (SNC) experiments. **Results:** Autoclavation partially nullified and partially modified the preparation's effects in the bioassay. The significant main treatment effects observed in the untreated condition for two outcome parameters were absent following autoclavation. By contrast, time-modulated day × treatment interactions were preserved in the remaining two parameters, and in one case strengthened. A correlation analysis revealed that the day-to-day pattern of the preserved effects was entirely decoupled from the untreated condition, indicating that autoclavation produced a qualitatively different temporal response pattern rather than preserving the original one. SNC experiments were stable throughout, confirming experimental reliability and the specificity of the observed effects. **Conclusions:** These findings contrast with those of the companion microwave study, in which all four outcome parameters were nullified, which seems attributable to non-thermal electromagnetic influences given the negligible thermal heating of the samples ($\Delta T \approx 0.091$ K). The qualitatively different outcome observed here suggests that thermal and electromagnetic stressors interact with potentised homeopathic preparations through distinct mechanisms. These findings cannot be extrapolated to other preparations or biological systems but warrant further investigation into the influence of standard manufacturing processes on the bioassay response of potentised homeopathic preparations.

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Keywords: autoclavation; steam sterilisation; ultra-molecular homeopathic preparations; *Lepidium sativum* bioassay; systematic negative control experiments; time-modulated treatment effects

1. Introduction

Sterilisation is a mandatory step in the manufacturing of sterile pharmaceutical preparations, including parenterals and ophthalmic preparations, where microbial contamination poses a direct risk to patient safety. The European Pharmacopoeia general text 5.1.1 establishes a clear hierarchy of sterilisation methods; terminal sterilisation by moist heat (steam sterilisation, autoclavation) is the preferred approach whenever the formulation permits, with aseptic processing or membrane filtration reserved for preparations that cannot tolerate heat or irradiation [1,2]. The reference conditions for steam sterilisation (121 °C for a minimum of 15 min, resulting in a sterility assurance level equivalent to a probability of no more than one non-sterile unit per million sterilised items) reflect the broad-spectrum efficacy of moist heat against microbial contaminants, including bacterial spores. These conditions are also compatible with the aqueous, closed-container formats typical of injectable and ophthalmic preparations [2]. Membrane filtration, while effective at removing microorganisms, operates by physical exclusion rather than inactivation, and is applicable only where thermal methods are contraindicated.

Homeopathic preparations intended for parenteral or ophthalmic use are subject to the same sterility requirements as their conventional counterparts. Regulations 11 and 15 of the German Homeopathic Pharmacopoeia (HAB) cover liquid potencies for injection and eye drops, respectively, and mandate compliance with the corresponding Ph. Eur. monographs. This places these preparations within the same regulatory sterilisation framework [3,4].

In this context, an important question must be addressed: do the thermal conditions of autoclavation alter the response of potentised homeopathic preparations in biological test systems?

For conventional pharmaceuticals, heat sensitivity is assessed in terms of chemical degradation of an identifiable active substance. At ultra-high dilution levels (in the present study a nominal dilution of 10^{-30} of the original substance, i.e., well beyond Avogadro's limit), no such substance is present in measurable quantities. Whether such preparations have specific effects is disputed [5], although plant-based basic research has reported effects in several model systems [6]. Standard stability testing is therefore uninformative for these preparations, and whether autoclavation affects their response in biological test systems remains an open question, with direct relevance to manufacturing practice.

Only a small number of studies have examined the effects of heat as physical factor on the bioassay response of potentised homeopathic preparations. Pupulin [7] reported that autoclavation of a *Sulphur* 14cH preparation altered its effect in a sorghum germination bioassay, producing a stimulatory rather than inhibitory response compared to non-autoclaved preparations of the same substance. However, as the study was published in abstract form only, full statistical reporting is absent and no systematic negative controls (SNCs) were employed, thus limiting the conclusions that can be drawn. Brizzi et al. [8] reported that the effect of As_2O_3 45x in a wheat germination model was unaltered at 20 and 40 °C, increased at 70 °C and decreased at 100 °C (5 min), without testing autoclave conditions. These findings, while limited in number, indicate that application of heat energy can interact with potentised preparations in ways that are not straightforwardly predictable.

We recently reported that exposure of aqueous *Stannum metallicum* 30x to close-proximity pulsed electromagnetic radiation in the GHz range, generated by a 2G cell phone over a period of 14 h, resulted in inactivation of the preparation's response across all

four outcome parameters of the bioassay employed [9]. Thermal heating of the samples under those conditions was estimated to be negligible ($\Delta T \approx 0.091$ K), indicating that the observed inactivation was attributable to non-thermal electromagnetic effects rather than heat. Autoclavation, by contrast, involves substantial thermal energy transfer at elevated pressure, raising the question of whether it alters the response of a potentised preparation in the bioassay, and whether its consequences differ from those of electromagnetic exposure. Crucially, the autoclavation data reported in the present study were obtained within the same experimental series as those of the cell phone study. Each experiment included untreated, cell phone-irradiated, and autoclave-treated preparations, assessed in parallel, using identical sample sets and experimental conditions. The untreated *Stannum metallicum* 30x data reported previously thus serve directly as the baseline for the present analysis, providing a well-controlled basis for comparing the effects of two physical factors on the same preparation.

The present study investigated whether autoclavation of *Stannum metallicum* 30x modifies its effects in a previously validated *Lepidium sativum* $CuCl_2$ bioassay [10–12], using SNC experiments to monitor experimental stability throughout.

2. Materials and Methods

2.1. General Experimental Procedure

The general experimental procedure was carried out according to Doesburg et al. [11]. *Lepidium sativum* seeds were germinated and grown for 96 h at 18 °C (±1 °C) in suspended plastic zipper bags on chromatographic paper moistened with homeopathic or control solutions. Following the growth period, seedlings were photographed for subsequent length determination, then crushed and extracted in the corresponding solutions. Extracts were mixed with a $CuCl_2$ dihydrate solution, pipetted into Petri dishes, and allowed to evaporate under standardised conditions, resulting in two-dimensional dendritic crystallisation patterns. Crystallisation patterns were evaluated by computerised image analysis. Experiments were performed in parallel by two independent laboratories in Denmark and the Netherlands (BRAD = Biodynamic Research Association Denmark; CL = Crystal Lab) and were conducted in a randomised and blinded fashion. The code was not revealed until data analysis was complete. For a schematic representation of a verum experiment, see Figure 1 (reprinted from [9], with permission).

2.2. Experimental Design

Ten verum experiments were performed in parallel in two independent laboratories (BRAD, CL; $n = 10 + 10$ sub-experiments), assessing 10 independently prepared sets of *Stannum metallicum* 30x and lactose 30x preparations. In each experiment, the two preparations were examined untreated, subjected to cell phone irradiation, or subjected to autoclavation. Data for the untreated and cell phone irradiated samples were reported previously [9,11,12]; the data of the untreated samples are reproduced here for comparison. In addition, 10 independent SNC experiments were performed in parallel in both laboratories ($n = 10 + 10$ sub-experiments), in which 6 preparations of distilled H_2O 1x each, produced as 10 independent sample sets, were evaluated. Data analysis was performed either at the level of experimental number ($n = 10$, allowing inclusion of the factor laboratory in the ANOVA F-tests) or at the level of experimental day ($n = 20$). Blinding and randomisation were performed at two levels for all experiments, with respect to the experimental series (verum or SNC) and with respect to the six individual treatments within each experiment. After the growth period, the bags were photographed, six cress extracts were produced, mixed with a $CuCl_2$ dihydrate solution, and allowed to crystallise under defined conditions (29 °C; initial rH 49%; air velocity 4 cm s^{-1}; HygroClip S, Rotronic

AG, Bassersdorf, Switzerland). Crystallisation experiments were carried out in six-fold repetition, resulting in 36 crystallisation plates per experimental day. The experimental design, incorporating 20 SNC experiments alongside the 20 verum experiments, allowed reproducibility and experimental stability of the bioassay to be monitored throughout the study, in accordance with established methodological standards for preclinical homeopathic potency research [13,14]. Further methodological details are reported elsewhere [11].

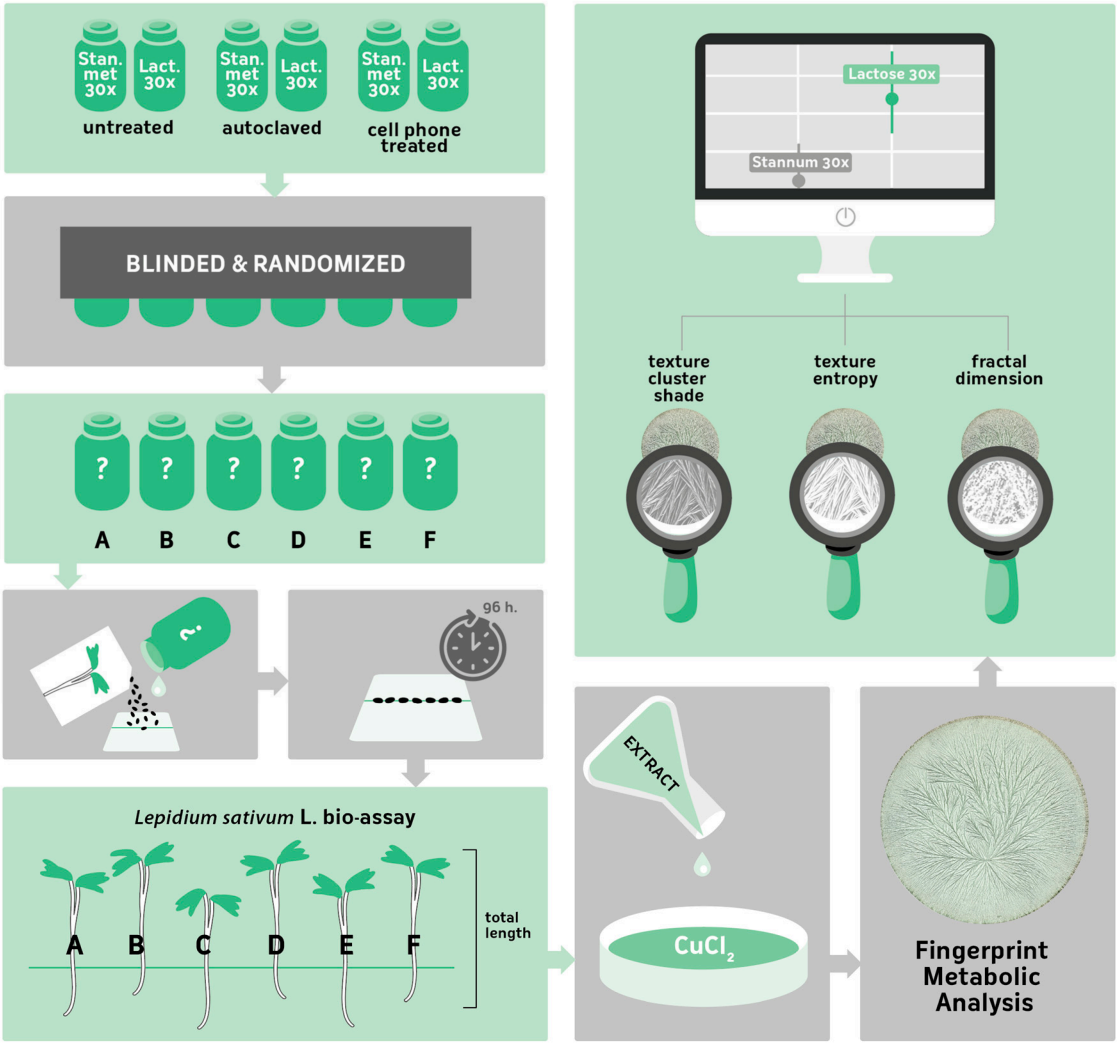


Figure 1. General design of a verum experiment. (1) Homeopathic potencies of *Stannum metallicum* 30x and lactose 30x were prepared and left untreated, subjected to cell phone irradiation, or autoclaved. (2) The six homeopathic potencies were randomised and coded A–F. (3) The preparations were used to grow cress seedlings by wetting chromatographic paper in plastic zipper bags for each of the six experimental conditions. (4) After a growth period of 96 h, the bags were photographed and an aqueous extract was prepared from crushed seedlings extracted in the corresponding potencies. Six crystallisation plates were prepared for each experimental condition. (5) The scanned crystallisation plates were subjected to computerised image analysis. Reproduced from [9] under CC BY 4.0.

2.3. Homeopathic Preparations

All liquid potencies and controls were prepared under sterile conditions (Laminar Flow Cabinet HFX-180, Skan, Basel, Switzerland, located in a Class C clean room) by author CS. Potentisation of the verum samples up to the fourth potentisation step was performed in solid medium by triturating one part of *Stannum metallicum* or lactose in nine parts of

lactose monohydrate as dilution medium (both 4x triturations supplied by Weleda AG, Schwäbisch Gmünd, Germany), according to manufacturing method no. 6 of the German Homeopathic Pharmacopoeia [15]. Subsequent dilutions were performed in liquid medium using autoclaved distilled water as diluent. In total, the procedure of 1:9 dilution with trituration or succussion was repeated 30 times, yielding a nominal dilution of 10^{-30} of the original substance. The resulting 30x potency preparations were distributed into 15 × 50 mL laboratory bottles per substance. Verum samples were subsequently divided into three groups for the three treatment conditions. Samples were wrapped in aluminium foil, coded, and stored in cardboard boxes at 18 °C until shipment to the crystallisation laboratories. The SNC solutions consisted of distilled autoclaved water succussed once (water 1x). These solutions were subsequently wrapped in aluminium foil, coded, and stored under the same conditions as the verum samples. For further detail on preparation procedures, see [11].

2.4. Autoclavation

Autoclavation of the homeopathic samples (*Stannum metallicum* 30x and lactose 30x) was performed by author CS. Prior to autoclavation, bottles were unwrapped from their aluminium foil covering and lids were positioned slightly ajar to allow steam penetration and pressure equalisation during the autoclave cycle. One third of the verum samples were autoclaved at 121 °C for 20 min (HST 6-6-6, Zirbus Technology AG, Bad Grund, Germany). Following the procedure, lids were sealed and bottles were returned to their aluminium foil wrapping before storage and shipment to the crystallisation laboratories under conditions identical to those of the untreated samples.

2.5. Cress Length Measurements

After 96 h of germination, seedlings were photographed in their plastic zipper bags alongside millimetre paper to calibrate subsequent length measurements. Lateral LED illumination and a black background were used to enhance contrast between seedlings and chromatographic paper. True curve length of the plants was measured using the software "Tracking 0.2.6" (A. Fritschy, Informatik-Lösungen, Zürich, Switzerland) and a Summagraphics SummaSketch III digitising tablet (GTCO CalComp, Columbia, SC, USA) connected to an Apple iBook G3 computer (Apple, Cupertino, CA, USA). Further details are reported elsewhere [11].

2.6. Computerised Image Analysis

Computerised image analysis was performed as described in detail elsewhere [11]. Briefly, crystallisation plate scanning and grey level co-occurrence matrix (GLCM) transformation were carried out as previously described, with circular regions of interest (ROIs) selected for texture analysis (TA). TA variable cluster_shade quantifies the degree of asymmetry in the GLCM, with higher values indicating greater asymmetry; entropy measures the randomness of grey level intensity distribution, with higher values indicating greater disorder. The mean local connected fractal dimension (LCFD) estimates the self-similarity of crystallisation patterns across spatial scales, calculated as the total number of pixels connected to seed pixels in a series of increasing oval regions of interest centred around each seed pixel [12].

2.7. Missing Data

Of a total of 1440 crystallisation plates prepared across all three treatment arms, 14 (< 1%) were discarded due to technical failures, primarily caused by solution leakage beneath the surrounding acrylic ring. For the autoclavation data reported in the present

study, 239 verum and 237 SNC plates were available for computerised analysis; for the untreated data used as baseline, 238 verum and 237 SNC plates were available.

2.8. Skewness Correction

Cress length data exhibited considerable left skewness (−1.46), corrected by exclusion of underdeveloped seedlings below 0.8 times the first quartile (15.7% data reduction), reducing skewness to −0.36 [9]. LCFD data exhibited right skewness (0.95), corrected by trimming values above the upper Tukey fence (4.42% data reduction), reducing skewness to 0.27 [9]. Texture analysis data exhibited no discernible skewness and were left untreated.

2.9. Data Evaluation and Statistics

Data evaluation was based on ANOVA F-tests performed with Statistica 6.0 for Windows (StatSoft Inc., Tulsa, OK, USA). Pairwise comparisons were evaluated with the LSD test, applied only when the preceding global F-test was significant ($p < 0.05$), constituting a protected Fisher's LSD procedure [16]. Graphs were plotted using RStudio version 2026.06.0+242 "Blue Plumbago" [17] and the packages "ggplot2" version 3.2.1 [18] and "gridExtra" version 2.3.

3. Results

3.1. Untreated Samples

Data for the untreated *Stannum metallicum* 30x samples were reported previously [9,12] and are reproduced here for comparison. In both the untreated and autoclave-treated conditions, all parameters exhibited highly significant differences in absolute values between the 20 experimental days ($p < 0.0001$, Table 1, main effect 1), reflecting the consistent day-to-day variation characteristic of this bioassay; subsequent results therefore focus on treatment effects and treatment-involving interactions. In the two-way ANOVA that focuses on the variability introduced by the 20 single experimental days, untreated *Stannum metallicum* 30x exhibited a significant main treatment effect for cluster_shade ($p = 0.034$, Table 1, main effect 2) and LCFD ($p = 0.011$, Table 1, main effect 2), and a significant day × treatment interaction for cress length ($p = 0.001$, Table 1, interaction 1–2) and entropy ($p = 0.024$, Table 1, interaction 1–2), indicative of a time-modulated treatment effect for the latter two parameters. No significant effects were observed in any of the SNC evaluations, indicating a stable experimental setup and a reliable and specific treatment effect [9,12].

The three-way ANOVA, mainly investigating the variability introduced by the 10 independent production batches and the two laboratories, further characterised these effects. For cress length, significant interactions were observed between laboratory and potency treatment ($p = 0.026$, Table 2a, interaction 1–3) and between laboratory, experimental number, and potency treatment ($p = 0.004$, Table 2a, interaction 1–2–3), indicating that the treatment effect differed between laboratories both in its overall magnitude and in its day-to-day temporal pattern. For cluster_shade, the significant main treatment effect ($p = 0.034$, Table 2a, main effect 3) was accompanied by a significant interaction between experimental number and potency treatment ($p = 0.041$, Table 2a, interaction 2–3). For entropy, a significant three-way interaction between laboratory, experimental number, and potency treatment was observed ($p = 0.003$, Table 2a, interaction 1–2–3), indicating that the time-modulated entropy effect was additionally differentiated by laboratory. For LCFD, the significant main treatment effect ($p = 0.012$, Table 2a, main effect 3) was not accompanied by any significant treatment-involving interactions.

Table 1. Results from two-way ANOVA F-tests for cress length, the TA variables cluster_shade and entropy, and LCFD (main effects and interaction). Independent experimental parameters were (1) Exp. Day = experimental day {1–20; $n = 20$}, and (2) treatment {Stannum met. 30x or lactose 30x (verum experiments), or water 1x and water 1x (SNC experiments), respectively; $n = 2$}. Each experimental parameter combination (statistical treatment cell) was assessed by approximately 160 seedlings (cress length), or six crystallisation patterns (cluster_shade, entropy and LCFD). Significant effects below $p < 0.05$ are shown in bold red font. Grey background colour indicates verum, and SNC results for the untreated samples. White background colour indicates verum, and SNC results for the autoclavation-treated samples.

Parameter	Verum Experiments			SNC Experiments		
	Main Effects		Effect Interactions	Main Effects		Effect Interactions
	(1) Exp. Day	(2) Treatment	1–2	(1) Exp. Day	(2) Treatment	1–2
	Untreated verum experiments (p-value)			SNC experiments (p-value)		
Cress length	**<0.0001**	0.2288	**0.0011**	**<0.0001**	0.8100	0.8083
Cluster_shade	**<0.0001**	**0.0336**	0.0647	**<0.0001**	0.2397	0.1401
Entropy	**<0.0001**	0.8826	**0.0242**	**<0.0001**	0.3321	0.2582
LCFD	**<0.0001**	**0.0112**	0.0565	**0.0050**	0.7137	0.2096
	Autoclavation-treated verum experiments (p-value)			SNC experiments (p-value)		
Cress length	**<0.0001**	0.7238	**0.0124**	**<0.0001**	0.2841	0.3580
Cluster_shade	**<0.0001**	0.4566	0.8861	**<0.0001**	0.3734	0.3596
Entropy	**<0.0001**	0.3342	**0.0008**	**<0.0001**	0.4562	0.7839
LCFD	**<0.0001**	0.2690	**0.0368**	**<0.0001**	0.7959	0.2233

Table 2. (**a**) and (**b**). Results from three-way ANOVA F-tests for the cress length, the TA variables cluster_shade and entropy and LCFD (main effects and interactions). Independent experimental parameters were (1) Lab = laboratory {BRAD, CL; $n = 2$}, (2) Exp. Num. = experimental number (production batch) {1–10; $n = 10$}, and (3) treatment {Stannum met. 30x or lactose 30x (verum experiments), or water 1x and water 1x (SNC experiments), respectively; $n = 2$}. Each experimental parameter combination (statistical treatment cell) was assessed by approximately 160 seedlings (cress length), or six crystallisation patterns (cluster_shade, entropy and LCFD). Significant effects below $p < 0.05$ are shown in bold red font. Grey background colour indicates verum and SNC results for the untreated samples. White background colour indicates verum and SNC results for the autoclavation-treated samples.

(**a**)

Parameter	Main Effects			Effect Interactions			
	(1) Lab	(2) Exp. Num.	(3) Treatment	1–2	1–3	2–3	1–2–3
	Untreated verum experiments (p-value)						
Cress length	**<0.0001**	**<0.0001**	0.2288	**<0.0001**	**0.0263**	0.1184	**0.0043**
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**
LCFD	0.2490	**<0.0001**	**0.0118**	**0.0011**	0.2462	0.0591	0.1575
	Autoclavation-treated verum experiments (p-value)						
Cress length	**<0.0001**	**<0.0001**	0.7238	**<0.0001**	0.7200	0.4019	**0.0014**
Cluster_shade	**<0.0001**	**0.0004**	0.4566	**0.0002**	0.7398	0.7004	0.8013
Entropy	**<0.0001**	0.0697	0.3342	0.1139	**0.0017**	**0.0105**	0.1136
LCFD	0.1332	**0.0002**	0.2690	**0.0125**	0.5934	0.6047	**0.0068**

Table 2. *Cont.*

(b)							
Parameter	**Main Effects**			**Effect Interactions**			
	(1) Lab	**(2) Exp. Num.**	**(3) Treatment**	**1–2**	**1–3**	**2–3**	**1–2–3**
SNC experiments corresponding to untreated verum experiments (*p*-value)							
Cress length	**<0.0001**	**<0.0001**	0.8100	**<0.0001**	0.1808	0.6409	0.8536
Cluster_shade	**<0.0001**	**<0.0001**	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
LCFD	0.5056	**0.0091**	0.7137	**0.0386**	0.2488	0.2472	0.1999
SNC experiments corresponding to autoclavation-treated verum experiments (*p*-value)							
Cress length	**<0.0001**	**<0.0001**	0.2841	**<0.0001**	**0.0380**	0.8122	0.2735
Cluster_shade	**<0.0001**	**0.0004**	0.3734	**0.0089**	0.3512	0.1541	0.6921
Entropy	**<0.0001**	**0.0257**	0.4562	**<0.0001**	0.7248	0.4757	0.8183
LCFD	0.1908	**0.0021**	0.7959	**<0.0001**	0.5574	0.0845	0.5280

Across almost all parameters and conditions, a significant interaction between laboratory and experimental number was consistently observed in both verum and SNC experiments (Table 2a,b, interaction 1–2), reflecting the inherent day-to-day variability between laboratories that is a well-established characteristic of this bioassay [9].

The corresponding SNC experiments showed no significant treatment effects or treatment-involving interactions (Table 2b) apart from a single lab–treatment interaction (cress length, $p = 0.038$), still basically confirming experimental stability with respect to the treatment factor.

3.2. Cress Length

A significant interaction was found between experimental day and potency treatment for the autoclavation-treated cress length data ($p = 0.012$, Table 1, interaction 1–2), indicative of a time-modulated potency treatment effect. This interaction was absent in the corresponding SNC experiments ($p = 0.358$, Table 1, interaction 1–2), corroborating the specificity of the observed treatment effect. The time-modulated treatment effect for cress length was thus preserved following autoclavation of the preparations (Figure 2B).

The three-way interaction between laboratory, experimental number, and potency treatment remained significant ($p = 0.001$, Table 2a, interaction 1–2–3), consistent with the untreated condition ($p = 0.004$), indicating that the time-modulated treatment effect was similarly differentiated by laboratory following autoclavation. Notably, the laboratory × treatment interaction observed in the untreated condition ($p = 0.026$, Table 2a, interaction 1–3) was absent following autoclavation ($p = 0.720$), suggesting that while the temporal laboratory differentiation of the effect was preserved, the overall between-laboratory difference in treatment magnitude was not. The corresponding SNC experiments were largely stable with respect to treatment-involving interactions (Table 2b), confirming experimental stability with respect to the treatment factor.

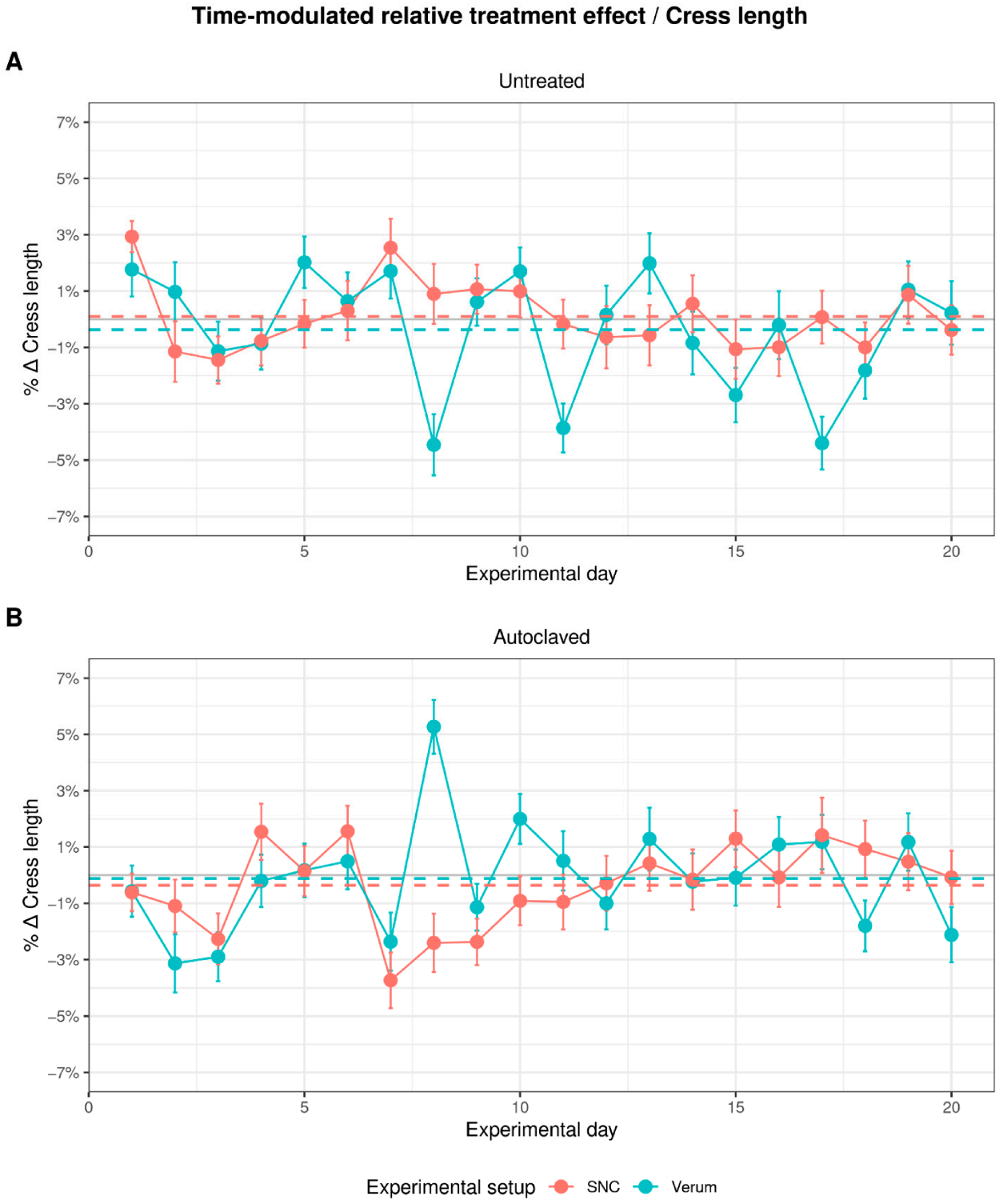


Figure 2. Time-modulated relative treatment effect for cress length across experimental days, expressed as 1 − (*Stannum metallicum* 30x/lactose 30x), such that a value of zero indicates no treatment effect and positive values indicate a reduction relative to lactose 30x. (**A**): Untreated preparations; (**B**): autoclave-treated preparations. Blue: verum experiments (*Stannum metallicum* 30x vs. lactose 30x); red: systematic negative control (SNC) experiments (water 1x vs. water 1x). Points represent mean values per experimental day ($n = 20$); error bars indicate standard error of the mean. Dashed horizontal lines indicate the grand mean across all experimental days for verum (blue) and SNC (red), respectively.

Comparing Figure 2A and Figure 2B, it is apparent that the treatment effect of *Stannum metallicum* 30x manifested on different experimental days in the untreated and autoclavation-treated conditions, pointing to a qualitative reorganisation of the temporal response pattern rather than its simple retention. To examine this observation more formally, a Spearman correlation analysis was performed between the per-day relative treatment effects of untreated and autoclaved preparations. For each experimental day, the relative treatment effect was computed as 1 − (*Stannum metallicum* 30x/lactose 30x), yielding a value of zero in the absence of a treatment effect, with positive values indicating a reduction relative to lactose. No significant correlation was found (Spearman $\rho = -0.092$, $p = 0.700$, $n = 20$; Figure 3A), confirming that the day-to-day pattern of the treatment effect was entirely decoupled between untreated and autoclaved preparations. The corresponding SNC analysis showed no significant correlation ($\rho = -0.301$, $p = 0.197$; Figure 3B), consistent with the expected random day-to-day variation in the absence of a treatment effect and confirming experimental stability. The considerably smaller scatter of data points in the SNC plot (Figure 3B) compared to the verum plot (Figure 3A) reflects the expected absence of day-to-day variation in the relative difference between two identical water preparations and underscores that the variability observed in the verum plot is driven by the time-modulated treatment effect rather than experimental noise.

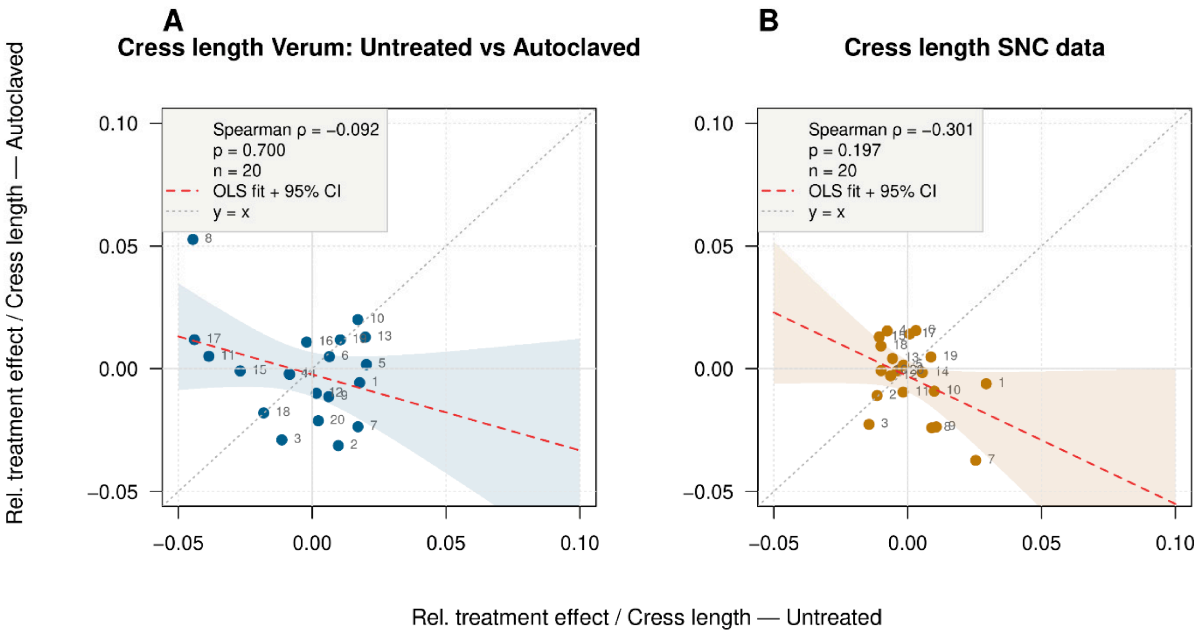


Figure 3. Spearman correlation analysis of the relative treatment effect on cress length between untreated and autoclavation-treated preparations. The relative treatment effect per experimental day was computed as 1 − (*Stannum metallicum* 30x/lactose 30x). (**A**): Verum experiments ($n = 20$); (**B**): systematic negative control (SNC) experiments ($n = 20$). The dashed grey line indicates the line of identity (y = x); the red dashed line shows the ordinary least squares (OLS) fit with 95% confidence interval. Individual experimental days are numbered.

3.3. TA Variable Cluster_Shade

The significant main treatment effect observed in the untreated samples ($p = 0.034$, Table 1, main effect 2) was absent following autoclavation ($p = 0.457$, Table 1, main effect 2). Figure 4A,B illustrates the impact of autoclavation on the cluster_shade treatment effect. The SNC experiments showed no significant treatment effects or treatment-involving interactions (Table 1), corroborating the specificity of the observed effect.

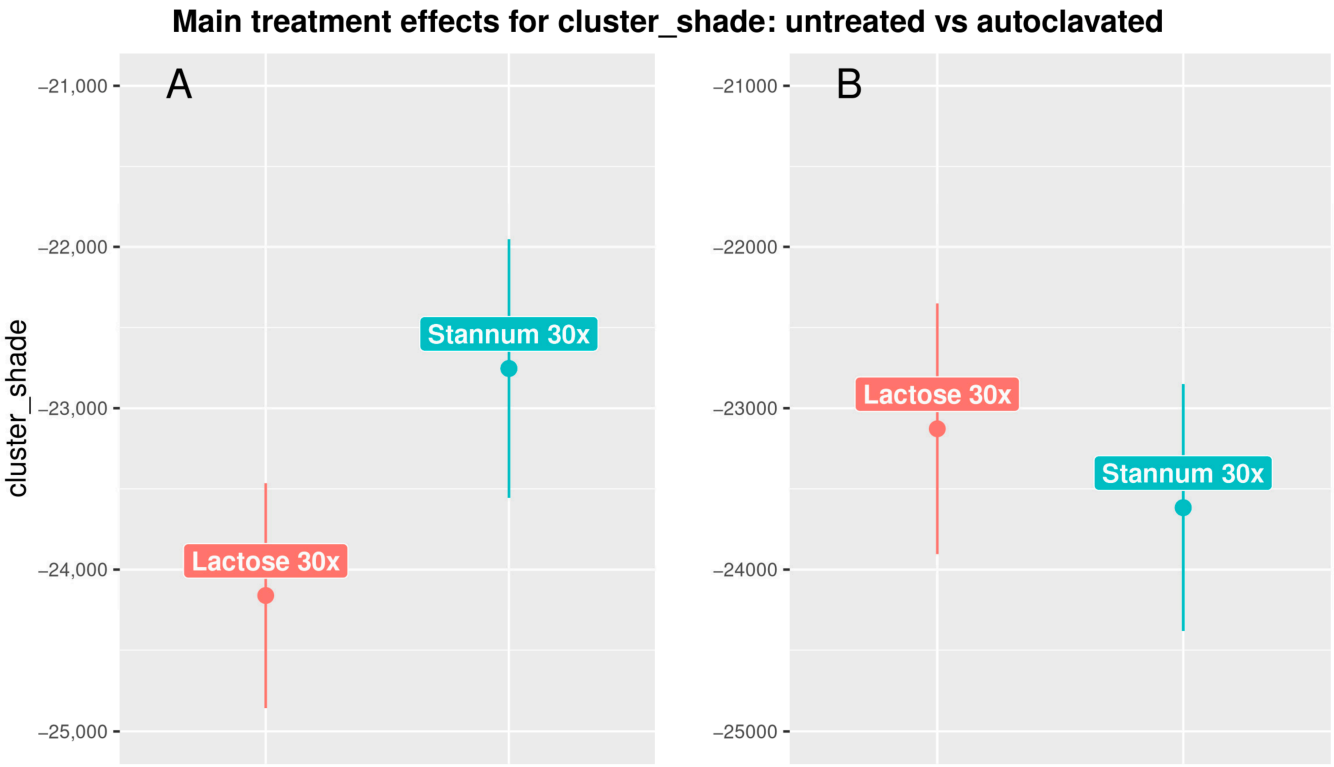


Figure 4. Mean ± standard error of the mean of cluster_shade of crystallisation patterns of Lepidium sativum seedlings germinated in *Stannum metallicum* 30x or lactose 30x. (**A**): cluster_shade, untreated preparations; (**B**): cluster_shade, autoclave-treated preparations. Values represent means across all crystallisation plates per preparation ($n \approx 238$ per treatment condition). Error bars indicate ± 1 standard error of the mean.

The three-way ANOVA confirmed the absence of a main treatment effect ($p = 0.457$, Table 2a, main effect 3) and the absence of any significant treatment-involving interactions following autoclavation. This contrasts with the untreated condition, in which a significant interaction between experimental number and potency treatment was observed ($p = 0.041$, Table 2a, interaction 2–3), suggesting that the time-dependent structure of the cluster_shade effect was also lost following autoclavation. The corresponding SNC experiments showed no significant treatment-involving interactions (Table 2b), confirming experimental stability with respect to the treatment factor.

3.4. TA Variable Entropy

The absence of a significant main treatment effect observed in the untreated samples ($p = 0.883$, Table 1, main effect 2) was consistent following autoclavation ($p = 0.334$, Table 1, main effect 2). The significant day × treatment interaction observed in the untreated samples ($p = 0.024$, Table 1, interaction 1–2) was preserved and indeed strengthened following autoclavation ($p = 0.0008$, Table 1, interaction 1–2), indicative of a persistent time-modulated potency treatment effect. The corresponding SNC experiments showed no significant treatment effects or treatment-involving interactions (Table 1), supporting the reliability of the verum finding. The time-modulated treatment effect for entropy was thus preserved following autoclavation of the preparations (Figure 5B).

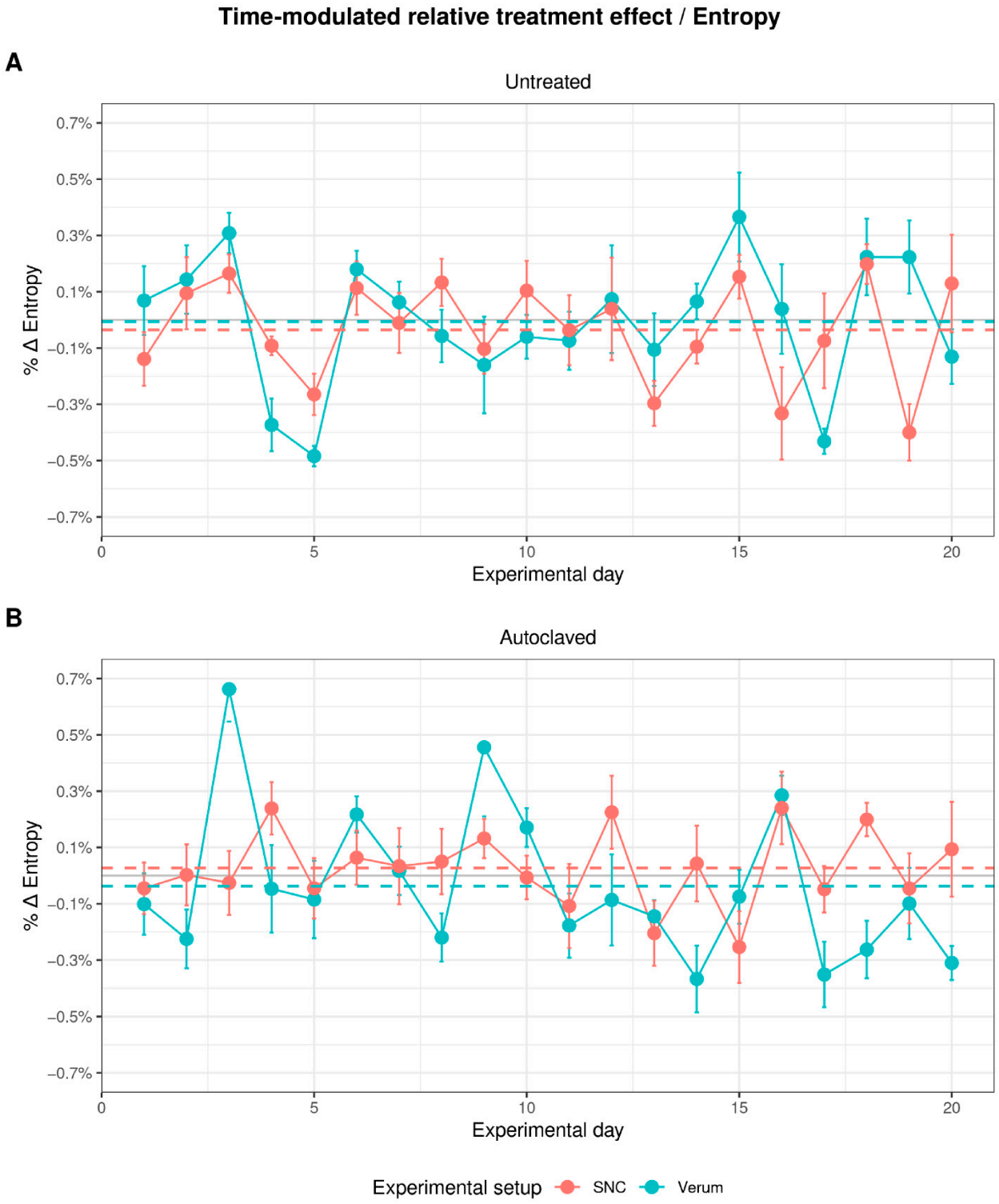


Figure 5. Time-modulated relative treatment effect for entropy across experimental days, expressed as 1 − (*Stannum metallicum* 30x/lactose 30x), such that a value of zero indicates no treatment effect and positive values indicate a reduction relative to lactose 30x. (**A**): untreated preparations; (**B**): autoclave-treated preparations. Blue: verum experiments (*Stannum metallicum* 30x vs. lactose 30x); red: systematic negative control (SNC) experiments (water 1x vs. water 1x). Points represent mean values per experimental day ($n = 20$); error bars indicate ± 1 standard error of the mean. Dashed horizontal lines indicate the grand mean across all experimental days for verum (blue) and SNC (red), respectively.

The three-way ANOVA revealed a reorganisation of the laboratory-dependent structure of the entropy effect following autoclavation. Whereas in the untreated condition, the entropy effect showed a significant three-way interaction between laboratory, experimental number, and potency treatment ($p = 0.003$, Table 2a, interaction 1–2–3), autoclavation nullified this three-way interaction ($p = 0.114$) while introducing two independent significant interactions. These were a laboratory × treatment interaction ($p = 0.002$, Table 2a, interaction 1–3), absent in the untreated condition ($p = 0.425$), and an experimental number × treatment interaction ($p = 0.011$, Table 2a, interaction 2–3), also absent in the untreated condition ($p = 0.598$). Importantly, the corresponding SNC experiments showed no significant treatment-involving interactions (Table 2b), confirming that this laboratory differentiation was specific to the verum treatment and not attributable to general experimental drift between laboratories.

Analogous to the approach employed for cress length, a correlation analysis was performed for entropy. No significant correlation was found between untreated and autoclaved preparations (Spearman ρ = 0.120, $p = 0.612$, $n = 20$; Figure 6A), consistent with the decoupling observed for cress length and with the structural reorganisation of the entropy effect identified in the three-way ANOVA. The corresponding SNC analysis likewise showed no significant correlation (ρ = 0.116, $p = 0.626$; Figure 6B), confirming experimental stability. As observed for cress length, the tighter clustering of SNC data points (Figure 6B) compared to the verum plot (Figure 6A) confirms that the day-to-day variability in the verum relative treatment effect is specific to the *Stannum metallicum* 30x preparation and not a reflection of general experimental variation.

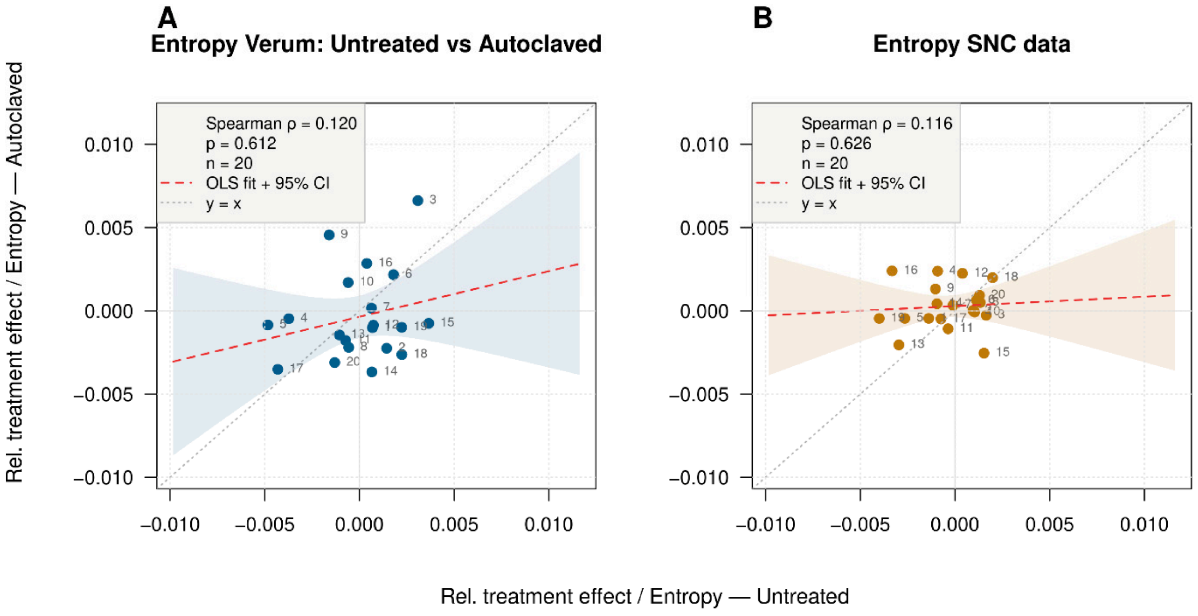


Figure 6. Spearman correlation analysis of the relative treatment effect for entropy between untreated and autoclavation-treated preparations. The relative treatment effect per experimental day was computed as 1 − (*Stannum metallicum* 30x/lactose 30x). (**A**): Verum experiments ($n = 20$); (**B**): systematic negative control (SNC) experiments ($n = 20$). The dashed grey line indicates the line of identity (y = x); the red dashed line shows the ordinary least squares (OLS) fit with 95% confidence interval. Individual experimental days are numbered.

3.5. LCFD

The significant main treatment effect observed in the untreated samples ($p = 0.011$, Table 1, main effect 2) was absent following autoclavation ($p = 0.269$, Table 1, main effect 2). A significant interaction between experimental day and potency treatment was observed

($p = 0.037$, Table 1, interaction 1–2), consistent with the trend observed in the untreated condition ($p = 0.057$, Table 1, interaction 1–2), which narrowly failed to reach significance at the 5% level. Figure 7A,B illustrate the impact of autoclavation on the LCFD treatment effect. The SNC experiments showed no significant treatment effects or treatment-involving interactions (Table 1), supporting the reliability of the verum finding.

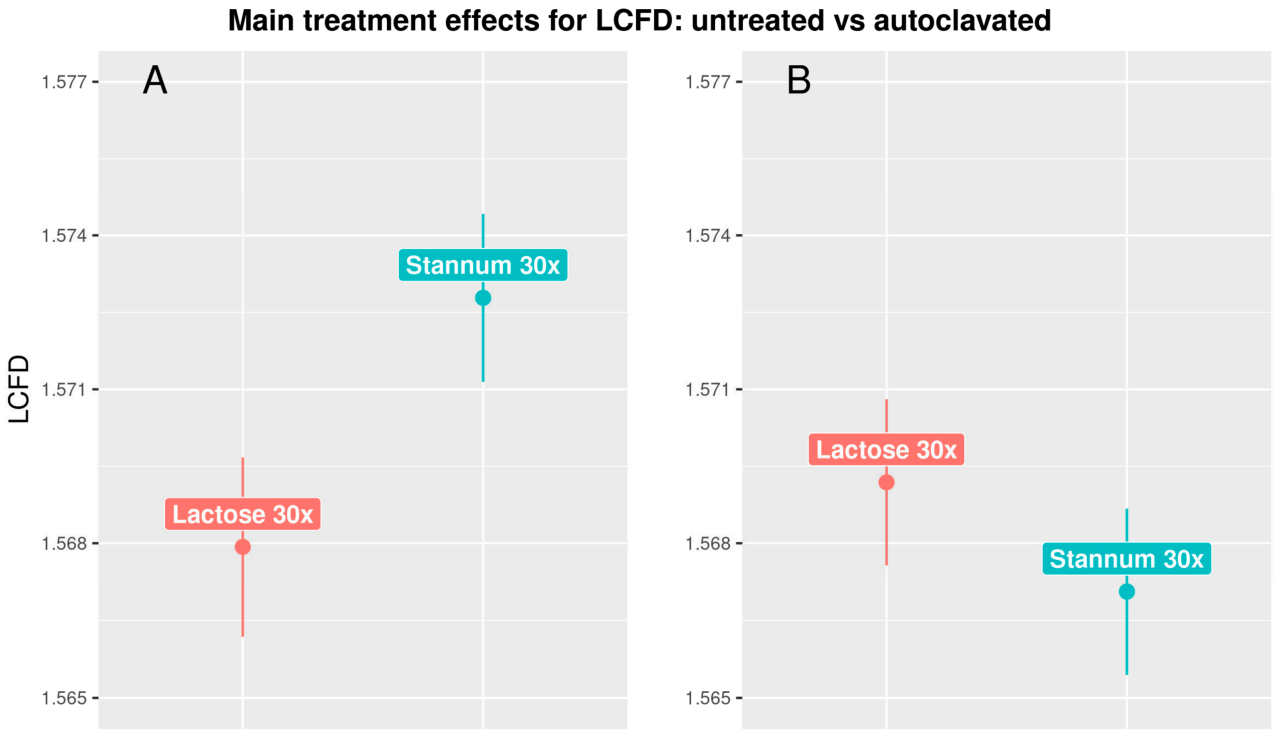


Figure 7. Mean ± standard error of the mean of LCFD of crystallisation patterns of Lepidium sativum seedlings germinated in *Stannum metallicum* 30x or lactose 30x. (**A**): LCFD, untreated preparations; (**B**): LCFD, autoclave-treated preparations. Values represent means across all crystallisation plates per preparation ($n \approx 238$ per treatment condition). Error bars indicate ± 1 standard error of the mean.

The three-way ANOVA confirmed the absence of a significant main treatment effect following autoclavation ($p = 0.269$, Table 2a, main effect 3). A significant three-way interaction between laboratory, experimental number, and potency treatment was observed ($p = 0.007$, Table 2a, interaction 1–2–3), absent in the untreated condition ($p = 0.158$), indicating that the marginal time-modulated interaction was additionally differentiated by laboratory following autoclavation. The corresponding SNC experiments showed no significant treatment-involving interactions (Table 2b), confirming experimental stability with respect to the treatment factor.

4. Discussion

4.1. Divergent Effects Across Outcome Parameters

The present study investigated whether autoclavation of *Stannum metallicum* 30x modifies its effects in the previously validated *Lepidium sativum* $CuCl_2$ bioassay. The results reveal a pattern that is a mixture between partial modification and partial nullification of the response of the preparation. The main treatment effects observed in the untreated condition for cluster_shade and LCFD were absent following autoclavation, while the time-modulated day × treatment interactions for cress length and entropy remained significant. However, a correlation analysis demonstrated that the day-to-day pattern of the relative treatment effect was entirely decoupled between untreated and autoclaved preparations for both parameters,

indicating that autoclavation produced a qualitatively different temporal response pattern rather than simply maintaining the original effect. Taken together, these findings suggest that autoclavation partially changed and partially nullified the response of the preparation in the bioassay used.

4.2. Comparison with the Application of Pulsed Microwaves

The present findings contrast markedly with those of the companion cell phone study, in which exposure of *Stannum metallicum* 30x to pulsed GHz electromagnetic radiation nullified the bioassay effects across all four outcome parameters [9]. In that study, the estimated thermal heating of the samples was negligible ($\Delta T \approx 0.091$ K), indicating that the observed inactivation was attributable to non-thermal electromagnetic effects. Autoclavation, by contrast, involves substantial thermal energy transfer at 121 °C for a minimum of 15 min, a physically distinct mechanism. The fact that these two physical treatments produce qualitatively different outcomes suggests that the electromagnetic and thermal components of the two interventions interact with the preparation through distinct pathways. Whereas non-thermal electromagnetic radiation appears sufficient to fully nullify the bioassay effects, thermal stress at autoclave conditions produces a more complex reorganisation of the response pattern.

4.3. Qualitative Change Rather than Nullification

The decoupling of the day-to-day treatment effect pattern between untreated and autoclaved preparations, as demonstrated by the correlation analysis, is an interesting finding of the present study. Despite the statistical preservation of time-modulated interactions in the two-way ANOVA for both cress length and entropy, the correlation between the per-day relative treatment effects of untreated and autoclaved preparations was negligible and not significant for both parameters (cress length: $\rho = -0.092$; entropy: $\rho = 0.120$). This indicates that the autoclaved preparation does not simply retain a weakened version of its original response signature; it exhibits a different one.

This finding is consistent with the only prior study investigating autoclavation effects on a potentised homeopathic preparation. Pupulin et al. [7] reported that autoclavation of a Sulphur 14cH preparation seemed to alter rather than nullify its effect in a sorghum germination bioassay, producing a stimulatory rather than inhibitory response compared to non-autoclaved preparations. That said, as noted, that study was published in abstract form only, without full statistical reporting or SNCs. The present study, using a rigorously validated bioassay with full SNC experiments throughout, provides more robust evidence pointing in the same direction: autoclavation qualitatively modified the response of a potentised homeopathic preparation in the bioassay used.

4.4. Reorganisation of the Entropy Effect

The three-way ANOVA revealed a reorganisation of the laboratory-dependent structure of the entropy effect following autoclavation. In the untreated condition, a significant three-way interaction between laboratory, experimental number, and potency treatment was observed ($p = 0.003$), indicating that the time-modulated entropy effect was differentiated by laboratory in a temporally structured way. Following autoclavation, this three-way interaction was nullified, while two independent significant interactions emerged: a laboratory × treatment interaction ($p = 0.002$) and an experimental number × treatment interaction ($p = 0.011$). The former indicates that the entropy treatment effect became laboratory-dependent following autoclavation in a way that was independent of time. This pattern was confirmed as verum-specific by the absence of any corresponding treatment-involving interactions in the SNC experiments. The source of this laboratory differentiation is unknown

and may reflect subtle differences in laboratory conditions like temperature, humidity, or crystallisation chamber characteristics that interact with whatever property of the preparation was modified by autoclavation. Notably, a similar but distinct laboratory-dependent structure was already present in the untreated entropy effect, suggesting that the bioassay's sensitivity to laboratory conditions is a real and persistent feature of this outcome parameter, rather than a technical artefact introduced by autoclavation.

4.5. Mechanistic Considerations

The mode of action of ultra-molecular homeopathic preparations remains unknown, and the present results do not resolve this question. In that light the mechanistic considerations below should be regarded as speculative. However, they provide empirical constraints on existing theoretical frameworks. One framework under current discussion proposes that coherent oscillations among water molecules, sustained through dipole interactions with local electromagnetic fields, may underlie the activity of potentised preparations [19–21]. The dipole nature of water renders it susceptible to external pulsed electromagnetic fields, which exert a torque on water dipole moments and may affect hydrogen bond networks [22,23]. Non-thermal pulsed electromagnetic radiation might disrupt these oscillations directly through field interactions, which is consistent with the nullification observed in the cell phone study [9]. Thermal energy, by contrast, acts on water structure through a fundamentally different mechanism, disrupting hydrogen bond networks through increased molecular kinetic energy [24,25].

The two stressors differ not only in their mode of energy transfer but also in the total energy deposited in the sample. Based on the specific absorption rate calculated for the cell phone study [9], the estimated energy absorbed per sample bottle during 14 h of irradiation was approximately 0.11 Wh, which is an upper bound. Autoclavation at 121 °C for 20 min, by contrast, requires approximately 6.5 Wh per bottle, approximately 57 times more. Autoclavation additionally involves elevated pressure. That a 57-fold greater energy input delivered through a physically broader mechanism results in a less complete nullification of the bioassay effects than pulsed electromagnetic radiation is striking. It suggests the preparation may be more susceptible to specific electromagnetic perturbation than to non-specific thermal disruption, consistent with the proposal that coherent water oscillations [19–21] are specifically sensitive to the polarised, pulsed character of the 2G signal [22,26]. The present study did not test water structure or electromagnetic coherence. In particular, the data cannot resolve whether thermal stress partially disrupts coherent water structures or generates a qualitatively different state altogether.

4.6. Multiple Comparisons and Limitations

The large number of statistical comparisons performed across parameters, conditions, and ANOVA types warrants caution in interpreting isolated borderline significant effects. The marginal day × treatment interaction observed for LCFD following autoclavation ($p = 0.037$) gains plausibility from its consistency with the trend already present in the untreated condition ($p = 0.057$) but should nonetheless be interpreted with restraint. Similarly, isolated borderline significant effects in the SNC data are within the range expected by chance given the number of tests performed and should not be over-interpreted. The exploratory nature of the correlation analysis comparing the day-to-day variability of the treatment effect for the untreated and autoclaved samples should also be noted; its findings should be regarded as hypothesis-generating pending independent replication.

4.7. Regulatory Implications

The present findings may have potential implications for the manufacturing of sterile homeopathic preparations. The European Pharmacopoeia general text 5.1.1 establishes terminal steam sterilisation as the preferred method for sterile aqueous preparations, with filtration reserved for heat-sensitive products [2]. Homeopathic preparations intended for parenteral or ophthalmic use are subject to the same sterility requirements [3,4], and potentised homeopathic ophthalmic preparations are terminally sterilised by autoclavation after the dynamisation process is complete. The present results suggest that this standard sterilisation step may alter the bioassay response of the preparation not by nullifying its response but by reorganising it in ways that are not predictable from the untreated preparation's behaviour. Whether this has clinical consequences or safety implications cannot be determined from the present preclinical bioassay data, and extrapolation to other preparations or biological systems is not warranted. Nevertheless, the question of whether terminally sterilised homeopathic preparations retain a bioassay response equivalent to that of the unsterilised preparation merits further investigation, particularly given that this assumption underlies current manufacturing practice.

4.8. Conclusions and Outlook

The source of the time-modulated treatment effects observed in this and previous studies remains unresolved. The decoupling of the day-to-day treatment effect pattern between untreated and autoclaved preparations adds a new dimension to this question, suggesting that the autoclavation procedure altered not only the magnitude but also the temporal structure of the preparation's response in the bioassay. Dedicated investigation of the factors underlying time modulation, including experimenter effects, seasonal variation in cress germination, and seed batch age, is currently underway in our laboratory and may contribute to a deeper understanding of both the phenomenon itself and the qualitative changes induced by autoclavation.

The present findings, taken together with those of the companion cell phone study [9], suggest that potentised homeopathic preparations may be sensitive to physical interventions in ways that the current pharmaceutical regulatory framework is not designed to detect or address. Both electromagnetic radiation and thermal energy produced measurable changes in the response of *Stannum metallicum* 30x–effects that are invisible to standard physicochemical stability testing. Whether these changes have clinical consequences cannot be determined from the present preclinical bioassay data, and extrapolation to other preparations or biological systems is not warranted. Nevertheless, the regulatory framework for sterile preparations [2] assumes that a sterilisation step meeting the sterility requirements leaves the preparation otherwise unchanged. Our findings suggest that this assumption might be inappropriate for potentised preparations.

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