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Biological effects and physico-chemical properties of extremely diluted aqueous solutions as a function of aging-time

Lucietta Betti*[a], Vittorio Elia[b], Elena Napoli[b], Grazia Trebbi[a], Michela Zurla[a], Daniele Nani[c], Maurizio Peruzzi[d] and Maurizio Brizzi[e]

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This study concerns the biological effects and physico-chemical variations of extremely diluted solutions (EDS) as a function of aging-time. The biological efficacy of As_2O_3 at the 45th decimal dilution/succussion (As 45×) was tested in a wheat germination model. Ten trials were carried out from 0 to 12 months after treatment preparation, using wheat seeds (*Triticum aestivum* L.) of the Pandas variety. Seeds were pre-treated by poisoning with 0.1% As_2O_3 solution to reduce germination, to allow a better evaluation of treatment effects. The outcome variable was the number of non-germinated seeds after 96 h. The As 45× treatment aged for less than three months did not show a significant effect on wheat germination, whereas when aged for longer (3–12 months) the effect became significant. Concerning the physico-chemical characteristics, specific conductivity of As 45× aged from 0 to 12 months was measured, using nine samples for each date. The results showed a clear increasing time trend of specific conductivity, more evident when considering the last three measurements, which correspond to more than six months of aging. The physico-chemical behavior of EDS strongly supports the significant biological effects observed in a wheat model.

Keywords: aging-time effect; arsenic trioxide; conductivity; extremely diluted solutions; wheat germination

Introduction

Extremely diluted aqueous solutions (EDS), currently used in homeopathic medicine, have been described as solutions obtained by diluting and rhythmically shaking (in the succussion or dynamization process) a mother tincture (Holandino et al. 2008; Czerlinski and Ypma 2010). At very high dilution levels (beyond the Avogadro limit) the probability of the presence of molecules of the original substance is near to zero (Marschollek et al. 2010). This aspect represents one of the reasons why the biological effectiveness of EDS is still controversial, although there is emerging evidence for their *in vitro* activity (Brizzi et al. 2000, 2011; Belon et al. 2004; Witt et al. 2007; Frenkel et al. 2010; Endler et al. 2011) as well as *in vivo* activity (Bellavite et al. 2006; Welles et al. 2007; Baumgartner et al. 2008; Shah-Rossi et al. 2009; Betti et al. 2010; Jäger et al. 2010; Magnani et al. 2010; Wyss et al. 2010). Based on physico-chemical measurements, it was hypothesized that the particular preparation technique of EDS could lead to a structural alteration of the aqueous solvent that appears to trigger the formation of molecular aggregates of water molecules (Samal and Geckeler 2001; Elia et al. 2004, 2010; Roy et al. 2005; Rao et al. 2007; Lo et al. 2009; Czerlinski and Ypma 2010; Elia and Napoli 2010). It was also hypothesized that the structure of hydrogen bonds in pure water is very different from that of aqueous EDS and not identical as expected (Rey 2003, 2007; Roy et al. 2005; Teixeira et al. 2006). Papers on water and aqueous solutions are increasingly featured in scientific literature (Chaplin 2011) but, as reported by Ball, 'no one really understands water' (Ball 2008, p. 291). Water constitutes a key element to the existence of all biological systems (Del Giudice et al. 2009) and behaves as a complex system, capable of auto-organizing as a consequence of small perturbations (Lobyshev et al. 2003; Lo et al. 2009). Many of the features reported in the works on the physico-chemical properties of water or EDS are not covered by current theories (Robinson et al. 2000; Errington and Debenedetti 2001; Bakker et al. 2005; Miranda 2008; Chikramane et al. 2010), but reference should be made to nonequilibrium thermodynamics (Prigogine 1977; Nicolis 1989; Kondepudi and Prigogine 1998).

The experience of our research group on this topic allowed us to simultaneously investigate both the biological

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effects and physico-chemical variations of EDS as a function of aging-time. In particular, the work of the Bologna University team (LB, GT, MZ and MB) centered on a series of experiments on an isopathic model, based on *in vitro* wheat germination and growth (Betti et al. 2010). According to 'isopathy', the same substance causing the disease can be used in low doses or high dilutions to treat the disease itself (Bellavite et al. 2007). In our model, a large number of wheat seeds were stressed with a sub-lethal dose of arsenic trioxide (As_2O_3) and then treated with decimal dilutions/succussions of the same substance: the consistency of the different statistical analyses and the reproducibility of most of the experimental results are notable (Betti et al. 2010). The Naples University research group (VE and EN) carried out systematic studies of the physico-chemical properties of EDS and have already shown that the process of iterated dilution and dynamization is able to permanently modify certain features of water. In particular, the measurement of specific conductivity was found to be extremely useful for tracking the structural alterations of the solvent water in response to various perturbations (Elia V, Elia L et al. 2007; Cacace et al. 2009; Elia and Napoli 2010; Elia et al. 2010).

In our previous papers, it was shown that the aging-time of test samples can affect both their biological efficacy (Brizzi et al. 2011) and their physico-chemical properties (Elia et al. 2006; Elia V, Elia L et al. 2008; Elia, Napoli et al. 2008). The aim of the study described here is to investigate, adopting the aforesaid isopathic model of *in vitro* wheat germination, whether there is a correlation between the biological effects and physico-chemical variations of EDS as a function of aging-time.

Materials and methods

Classes of treatment

Two classes of treatment were tested:

- undiluted/unsuccussed control (C): aqueous solution of sodium bicarbonate at 7.0×10^{-5} mol l^{-1} ($NaHCO_3$, Sigma-Aldrich, St. Louis, MO, USA).
- As 45×: arsenic trioxide at the 45th decimal dilution/succussion (As_2O_3, Sigma-Aldrich, St. Louis, MO, USA).
- The choice of an undiluted/unsuccussed control was based on our previous findings (Betti et al. 2000, 2010; Brizzi et al. 2000; Elia et al. 2004, 2005) and is explained in considerable detail in the 'Discussion and conclusions' section.

As 45× was obtained starting from 0.2% of As_2O_3 in twice-distilled and sterilized water through the iteration of two processes: dilution and succussion. In the present paper the dilution process (1:10) was performed using an aqueous solution of sodium bicarbonate of known concentration (7.0×10^{-5} mol l^{-1}) as 'solvent'. The rationale of using this solvent is related to the storage of test samples in Pyrex glass containers for long periods: as reported in a previous paper (Elia, Napoli et al. 2008), chemical impurities released by the glass affect electrical conductivity. These main impurities, measured in the range of ppm, are derived from alkaline oxides (Na_2O); in contact with water they transform into sodium hydroxide (NaOH) that, due to atmospheric carbon dioxide (CO_2), turns into sodium bicarbonate ($NaHCO_3$). The use of a $NaHCO_3$ solution at known concentration as a 'solvent' can overcome the problem of the different contributions of impurities to electrical conductivity (Elia V, Elia L et al. 2007; Elia, Napoli et al 2007; Belon et al. 2008). The succussion consisted in a violent shaking of the solution by means of a mechanical apparatus (DYNA HV 1 by Debofar N.V.S.A., Oostduinkerke, Belgium). In a single succussion process, 100 vertical strokes were applied at the frequency of 8.3 Hz to the Pyrex glass vessel (100 ml) filled to 50% capacity. A Hahnemannian multiple glass method was applied, using Pyrex glass vessels cleaned with standard laboratory procedures: glass containers were treated with a $H_2SO_4/K_2Cr_2O_7$ solution, then rinsed with twice-distilled water until the electric conductivity of the rinsing water reached a value of $1.2 \pm 0.2\,\mu S\ cm^{-1}$, the same as twice-distilled water (Elia et al. 2009). Dilution and succussion procedures occurred under sterile conditions. After their preparation the test samples were poured into Pyrex bottles and letter-coded (blinded) by a person not involved in the experiments. Codes were kept by independent people until disclosure. The test samples were stored at room temperature in the dark and used in subsequent experiments (both biological and physico-chemical) until an aging-time of 12 months was reached. A total of 18 Pyrex glass bottles (nine for the control and nine for As_2O_3 45×) were prepared both for biological and physico-chemical experiments; one bottle per treatment per experiment was used.

Biological model

The experiment was performed at the Association for Sensitive Crystallization, located in Nibbiano (Piacenza, Italy), and consisted of nine trials carried out from 0 to 12 months after treatment preparation (see aging-time in Table 1), using wheat seeds (*Triticum aestivum* L.) of the Pandas variety (Consorzio Agrario, Pavia, Italy). We focused our attention on seed germination *in vitro*, applying the same biological pattern employed in previous studies (Brizzi et al. 2011): we placed a fixed number of 36 seeds, selected for integrity, on sterilized sand in sterile plastic Petri dishes of 10-cm diameter; each trial consisted of 21 Petri dishes per treatment. At the beginning of each experiment, a standard quantity of treatment (20 ml) was pipetted into each dish, without disturbing the seeds. The Petri dishes were randomly distributed, following a circular pattern, in a germination box mounted on an electrically driven plate rotating at 90 rpm. Dishes were kept at room temperature

Table 1. Exploratory statistics and statistical inference of the wheat germination model.

Experiment	Aging-time (months)	n	Average value (X)		Standard deviation		Test statistic		Cumulative average	
			C	As 45×	C	As 45×	Poisson	Mann–Whitney	C	As 45×
1	0	21	6.57	5.38	2.18	2.65	ns	ns	6.57	5.38
2	1	21	5.52	4.95	1.72	2.20	ns	ns	6.05	5.17
3	2	21	5.81	5.28	2.71	1.83	ns	ns	5.97	5.20
4	3	21	6.57	4.28	2.21	2.10	2.29**	1.75*	6.12	4.97
5	4	21	6.29	4.57	2.83	2.46	2.38**	1.89*	6.15	4.89
6	5	21	6.62	5.19	2.80	3.08	1.90*	1.79*	6.23	4.94
7	6	21	7.10	4.62	2.99	2.38	3.32***	2.72**	6.35	4.90
8	8	21	6.90	4.24	3.13	3.04	3.66***	2.60**	6.42	4.81
9	12	21	6.29	3.81	3.10	2.75	3.57***	2.58**	6.41	4.70
Overall		189	6.41	4.70	2.67	2.53	7.03***	5.45***	—	—

Notes: n = sample size (number of Petri dishes); X = number of non-germinated seeds (out of 36); C = control; ns = not significant. * = significant at $p < 0.05$; ** = significant at $p < 0.01$; *** = significant at $p < 0.001$.

(20°C), in daylight and at a constantly high humidity rate (70% relative humidity). This procedure was followed to obtain maximum homogeneity of the experimental conditions. The number of non-germinated seeds after 96 hours in each standard experiment was then counted and recorded as the outcome variable (denoted with X).

Wheat seeds were pre-treated by 30 min of poisoning with 0.1% arsenic trioxide aqueous solution (As_2O_3, Sigma-Aldrich), then rinsed in tap water for 60 min, dried in ambient air until they reached 12% moisture content, and stored in the dark at room temperature until use. This stress reduced the germination rate by 15% with respect to the non-stressed control, obtaining a germination rate of ~80% (Brizzi et al. 2000, 2011).

Conductivity measurements

Systematic measurements of specific conductivity of the two classes of treatment were performed at the Department of Chemistry, University ‘Federico II’ of Naples. Conductivity data were collected using a conductometer, model 3200 by YSI, employing a conductivity cell with constant equal to $1.0\,\mathrm{cm}^{-1}$. The cell was periodically calibrated by determining the cell constant $K(\mathrm{cm}^{-1})$. The specific conductivity χ (μS cm^{-1}) is the product of the cell constant and the conductivity of the treatment. For a given conductivity measuring cell, the cell constant is determined by measuring the conductivity of a KCl solution with a specific conductivity known with great accuracy, at several concentrations and temperatures. All values of conductivity were measured in a room at controlled temperature of 25 ± 1°C, corrected to 25.0 °C, using a pre-stored temperature compensation for pure water (Light and Licht 1987). The probe was washed many times after each measurement, using twice-distilled water, until the conductivity reached the same value of twice-distilled water. For each date (see aging-time in Table 2) measurements were performed on nine samples both for the control and As 45×.

Statistical analysis

When analyzing seed germination, the main outcome variable was the number X of non-germinated seeds in a Petri

Table 2. Some exploratory statistics and statistical inference on conductivity (expressed in μS cm^{-1}).

Experiment	Aging-time (months)	n	Average value (X)		Standard deviation		Test statistic		Cumulative average	
			C	As 45×	C	As 45×	Poisson	Mann–Whitney	C	As 45×
1	0	9	6.67	6.90	0.19	0.12	3.17**	2.56**	6.67	6.90
2	1	9	7.01	7.51	0.22	0.10	6.24***	3.31***	6.84	7.21
3	2	9	6.73	7.57	0.18	0.16	10.61***	3.40***	6.80	7.33
4	3	9	6.84	7.58	0.17	0.09	11.75***	3.40***	6.81	7.39
5	4	9	6.95	7.59	0.23	0.07	7.91***	3.44***	6.84	7.43
6	5	9	6.93	7.69	0.16	0.11	11.71***	3.58***	6.85	7.47
7	6	9	6.90	7.80	0.21	0.19	9.55***	3.49***	6.86	7.52
8	8	9	6.61	8.77	0.22	0.40	14.04***	3.53***	6.83	7.68
9	12	9	7.07	8.91	0.26	0.46	10.57***	3.58***	6.86	7.81
Overall		81	6.86	7.81	0.21	0.23	27.89***	9.90***	—	—

Notes: n = number of measurements; X = conductivity in μS cm^{-1}; C = control; ns = not significant. ** = significant at $p < 0.01$; *** = significant at $p < 0.001$.

dish. As done in previous experimentations (Betti et al. 2010), we checked the goodness of fit of Poisson distribution by applying the Kolmogorov–Smirnov test (Stephens 1974), based on the comparison between the empirical cumulative distribution function and the theoretical one.

After confirming the goodness of fit, we made an overall comparison between the experiments, in a sort of 'Poissonian ANOVA', using the test proposed by Sachs (1984). Denoting the Poisson parameters to be compared $\lambda_1, \lambda_2, \ldots, \lambda_k$, the null hypothesis is $H_0 : \lambda_1 = \lambda_2 = \ldots = \lambda_k$; if x_i is the total number of non-germinated seeds in the ith sample and t_i is the total number of experiments of the same sample, we denote with

$$\hat{\lambda} = \frac{\sum_{i=1}^{k} \hat{\lambda}_i t_i}{\sum_{i=1}^{k} t_i} \tag{1}$$

the overall Poisson parameter estimate. Now, if we compute the values:

$$z_i = \begin{cases} 2(\sqrt{x_i + 1} - \sqrt{t_i \cdot \hat{\lambda}}) & if\,\hat{\lambda}_i < \hat{\lambda} \\ 2(\sqrt{x_i} - \sqrt{t_i \cdot \hat{\lambda}}) & if\,\hat{\lambda}_i \geq \hat{\lambda} \end{cases} \tag{2}$$

the global Poisson test statistic is the following:

$$w = \sum_{i=1}^{k} z_i^2 \cong (H_0 \;\; true) \cong \chi^2_{(k-1)}. \tag{3}$$

Therefore, the test can be performed with the aid of chi-squared tables, considering $k - 1$ degrees of freedom. The test is one-tailed on the right, so we reject H_0 only for large values of w.

After calculating, for each sample, the usual exploratory statistics such as average and standard deviation (SD), we compared treatment and control data by a parametric pairwise Poisson test (see Sachs 1984) and then by the Mann–Whitney rank sum test for independent samples (Conover 1980).

The pairwise Poisson test is based on the following test statistic:

$$\hat{z} = \frac{T_A - T_B - 1}{\sqrt{T_A + T_B}}, \tag{4}$$

where T_A and T_B are the total number of non-germinated seeds in the two samples compared. When the Poisson parameters compared are equivalent (null hypothesis), the test statistic follows a standard normal distribution.

The Mann–Whitney test is based on a global ranking of sample data; if the sum of ranks from one sample is considerably smaller (or greater) than the other sample's sum, the null hypothesis of equal level of magnitude is rejected. The test statistic is:

$$U = \min(R_A, R_B), \tag{5}$$

where R_A and R_B are the two sums of ranks, one corresponding to treatments, the other to controls. Parametric and non-parametric tests were applied because they use a different kind of sample information: the first one is based on the global number of germinated and non-germinated seeds and the other one on a ranking of the individual trial results. Therefore, it is important to check data in both ways to give good confirmation of the existing difference between treatment and control. Since the efficacy of homeopathic treatments is not a given fact, complete information is needed in order to state that the effects are significant.

To study the time trend of seed germination, we calculated the cumulative averages, starting from the first experiment and adding a further experiment for each step of time. The resulting data (one for each experiment) were joined with smoothed curves, obtained by using the Microsoft Excel graphic smoothed pattern. The cumulative trend for X was then summarized by least-squares linear interpolation, and the Bravais–Pearson linear coefficient of correlation $r = \frac{Cov(X,Y)}{SD(X)SD(Y)}$ was computed to determine the degree of linearity.

On the other hand, when studying conductivity, we considered that data were approximately unimodal and not too skewed, so that a normal approximation was suitable. As described above for germination data, we applied a set of exploratory statistics to conductivity data and a parametric statistical test (Student's t-test) as well as a non-parametric test (Mann–Whitney rank sum test). Here too, the double approach (parametric and non-parametric) gives us reliable confirmation of the treatment effect. We also calculated cumulative means for studying the effect of aging-time and, as for germination data, a linear interpolation of these cumulative means was plotted.

When comparing the results of the germination and conductivity experiments, we calculated the relative variation (RV) from the starting point (first experiment), obtained by applying the formula:

$$\mathrm{RV} = \left(\frac{x_t - x_0}{x_0}\right) 100, \tag{6}$$

where x_t is the experimental value at time t and x_0 is the starting value, corresponding to the first experiment.

Results

Biological effects on a wheat germination model

Negative control experiments

The variability of the test system was evaluated in a preliminary set of independent and systematic negative control experiments using the solvent ($NaHCO_3 7.0 \times 10^{-5}$ M) as the only test substance. First of all, we checked the goodness of fit of the Poisson model to a set of six independent experiments of 21 Petri dishes each, by using the Kolmogorov–Smirnov (KS) test: the reduced value of the KS statistic (0.055) led us to confirm Poisson distribution as a suitable model for germination data. We then performed a multiple Poisson test comparing the six experiments and found no significance whatsoever ($w = 1.47$, with five degrees of freedom), thereby ensuring that the experimental set-up was stable and did not generate false positive results.

Exploratory statistics, statistical inference and time trend analysis

We began our analysis by computing a set of exploratory statistics (Table 1) that provided an initial visual impression of how the studied treatments affected wheat germination. It can be clearly seen that the average number of non-germinated seeds (X) is higher in the control group, in all the experiments. If the whole set of data is considered, the average value of X in control group is 6.41 while in the As 45× group it is reduced to 4.70, with a decrease of 26.61%. In particular, to simplify the visual impression and interpretation of our results, Figure 1 shows the average number of germinated seeds normalized against the corresponding control values set equal to 100. We can see that the As 45× treatment induces a stimulating effect on germination, increasing significance in the last experiments. Comparing the standard deviations (Table 1), we can observe an overall slight reduction (−5.23%) in the As 45× treatment group with respect to the control; however, a sharp oscillatory trend can be observed between the experiments. We also indicate the aging-time of each experiment (ranging from 0 to 12 months), which will be useful when analyzing the time trend of the effects.

Before applying statistical inference, we checked again, as done in previous papers (Betti et al. 1994, 2010; Brizzi et al. 2000), the goodness of fit of Poisson distribution in the treated group (As 45×). By means of the Kolmogorov–Smirnov test we did not detect any significant discrepancy ($KS = 0.121$), confirming that the Poisson model is suitable for germination data. After this check, we compared overall samples (each consisting of 189 Petri dishes) in two different ways: applying a pairwise Poisson parametric test as well as a Mann–Whitney rank sum test (Table 1): in both cases the difference was strongly significant ($p < 0.001$), revealing a stimulating effect on germination of the As 45× treatment versus the control. We then performed the same tests comparing the As 45× and the control within each experiment, and the stimulating effect was significant only in the experiments carried out after three months following the treatment preparation (experiments 4–9), as reported in Table 1. We can also observe, although it is not at all surprising, that the parametric test, being statistically more powerful, gives lower p-values referring to the same samples, with respect to the non-parametric test based on ranks.

Reading Table 1 again, we can derive a first indication about the time trend of wheat germination due to the aging effect of treatments. Indeed, if we follow the mean values of X (non-germinated seeds) within the C and As 45× groups, respectively, in subsequent experiments (1 to 9) we find an oscillatory trend in the control group and a globally decreasing trend in the treated one. We also included cumulative means for smoother results, reducing random oscillations between single experiments. We have tried to provide a graphical representation of the time trend of germination using cumulative percentages of germinated seeds: in Figure 2 we plotted the observed cumulative percentages (A) and the corresponding least-squares linear interpolation (B). By looking at both figures, it is evident that the trend is oscillatory for the control group ($r = -0.361$), while it is clearly increasing and very near to linearity ($r = +0.904$) for the As 45× group.

Physico-chemical effects on conductivity

Exploratory statistics, statistical inference and time trend analysis

We began our analysis of conductivity by computing the average and standard deviation of each experimental sample, reported in Table 2. It is worth noting that the average conductivity in the control samples is almost constant as a function of time, whereas in the As 45× samples it shows a clearly increasing time trend. This increasing trend is more evident when considering the last three measurements,

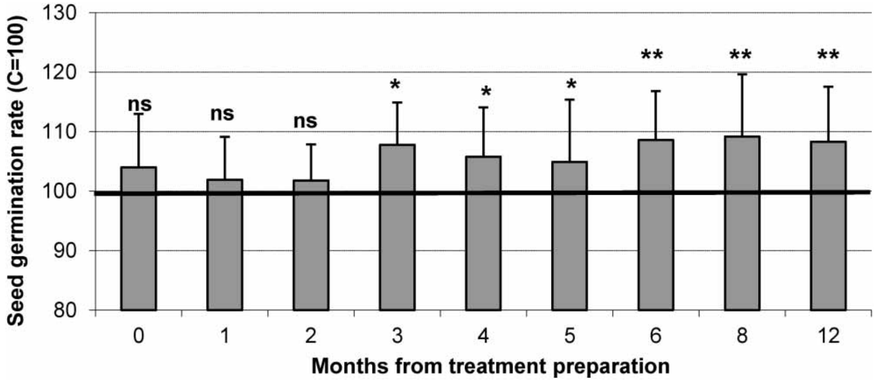

Figure 1. Average number of germinated seeds for subsequent experiments (control = 100); bars indicate standard deviations. ns = not significant; * = significant at $p < 0.05$; ** = significant at $p < 0.01$ (Mann–Whitney test).

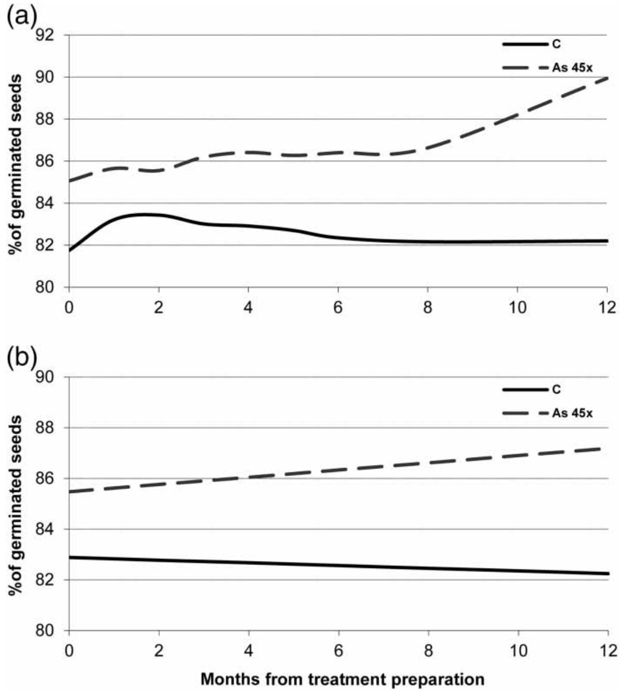

Figure 2. Trend of cumulative average percentages of germinated seeds: (A) observed cumulative average percentages; (B) linear interpolation.

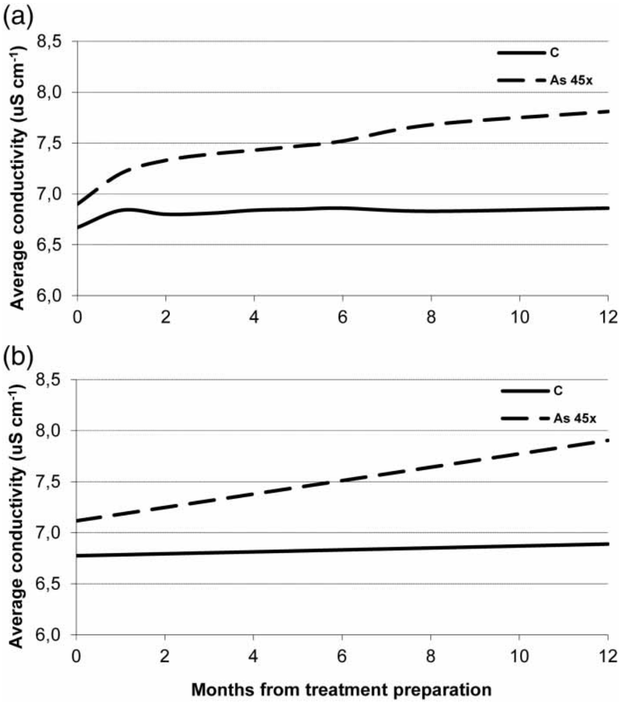

Figure 3. Trend of cumulative average conductivity: (A) observed cumulative average; (B) linear interpolation.

which correspond to more than six months of aging. Regarding inference results, it can be easily observed that the average difference of conductivity between control and treatment is always highly significant, following the parametric approach as well as the non-parametric one. We also computed the cumulative average conductivity, in order to reduce random oscillations between single measurements. In Figure 3, these cumulative average values are plotted (A) jointly with the corresponding least-squares linear interpolation (B): the difference between the almost oscillatory trend of control ($r = +0.605$) and the marked increasing trend of conductivity is visually evident in the As 45× group, which is near to perfect linearity ($r = +0.932$).

Comparison between biological and physico-chemical effects

We also attempted to compare the biological effects on wheat germination and physico-chemical effects on conductivity, as a function of aging-time from treatment preparation. Radar diagrams of the time trend of seed germination and conductivity are shown in Figures 4A and B, respectively. It is possible to note, for both variables, that the control remains rather homogeneous, while the treatment induces a marked increasing time effect. Moreover, Table 3 reports the relative variation of germinated seed percentage and the corresponding variation of conductivity, considering the result of the first experiment as a reference point. We can note that both variables (germination and conductivity)

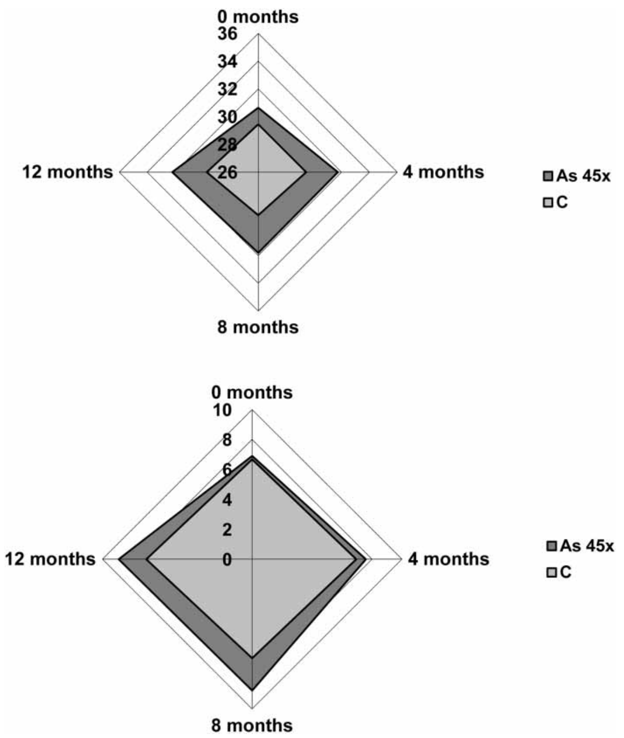

Figure 4. Radar diagrams showing the aging-time effect (0 to 12 months after treatment preparation) on seed germination (A) and conductivity (B). Germination is expressed in terms of germinated seeds (out of 36) and conductivity as μS cm^{-1}.

Table 3. Comparison between the effects of aging-time for germination (% of germinated seeds) and conductivity, by means of relative variation from the starting point.

Aging-time (months)	Relative variation (%)			
	Germination		Conductivity	
	C	As 45×	C	As 45×
1	+3.57	+1.40	+5.15	+8.84
2	+2.58	+0.33	+0.95	+9.71
3	0.00	+3.59	+2.60	+9.86
4	+0.97	+2.64	+4.25	+10.00
5	−0.17	+0.62	+3.95	+11.45
6	−1.78	+2.49	+3.50	+13.04
8	−1.14	+3.73	−0.85	+27.10
12	+0.97	+5.13	+6.05	+29.13

Note: C = control.

have an evident increasing trend in the As 45× group, although the increase of the relative variation is much sharper for conductivity, especially after more than six months of aging-time.

Discussion and conclusions

The results presented here seem to demonstrate a relationship between biological and physico-chemical effects of EDS as a function of aging-time. First of all, it is worth pointing out that, in this experimentation, undiluted/unsuccussed water was used as a control. This choice can have some implications in relation to the specificity of the measured effects: in fact, if the control is undiluted/unsuccussed, the difference between As 45× and the control could be ascribed to unspecific physicochemical alterations, due to the succussion procedure (Baumgartner et al. 1998). In our previous papers (Brizzi et al. 2000; Betti et al. 2010), we pointed out that, in wheat stressed seeds, even decimal dilutions/succussions of pure water (the solvent used for homeopathic treatment preparation) gave highly significant results with respect to an undiluted/unsuccused control. Nevertheless, the interaction of succussion and high dilution gave the most relevant results: the germination increase induced by As 45× was highly significant with respect to diluted/succussed water (H_20 45×) (Betti et al. 2000). As far as the physico-chemical properties are concerned, in our previous papers (Elia et al. 2004, 2005) higher values of the conductivity both in EDS and in diluted/succussed water with respect to undiluted/unsuccussed water (control) were shown; in this case too, the highest values of conductivity were generally obtained in EDS with respect to diluted/succussed water. These findings seem to indirectly highlight the existence of the real efficacy of homeopathic treatments, at least as concerns our wheat germination model and conductivity measurements. In any case, in order to separately detect the specific effects of the homeopathic treatment and of the succussion process only, in future investigations we will test both diluted/succussed and undiluted/unsuccussed controls, respectively (Stock-Schröer et al. 2009).

As far as EDS biological effects are concerned, the plant model presented here is based on the *in vitro* germination of wheat seeds stressed with a sub-lethal dose of As_2O_3 and then treated with decimal dilutions/succussions of the same substance. We adopted this 'isopathic approach' since, in previous studies, we had observed an 'isopathic sensitization', i.e. a considerable increase of treatment effect when working with stressed seeds (Brizzi et al. 2000, 2011). This behavior was confirmed in a recent review regarding the use of homeopathic preparations in experimental studies with abiotically stressed plants (Jäger et al. 2011), whereas in healthy plant tests the effects were less pronounced (Majewsky et al. 2009). The first general conclusion we can make on the germination results reported here is that the As 45× treatment induced a marked stimulating effect, in terms of an overall reduction in the number of non-germinated seeds (−26.6%). This percentage is consistent with the value (−21.2%) obtained at 20 °C in a recent work (Brizzi et al. 2011) and in previous studies on *in vitro* wheat germination and growth (Betti et al. 1997; Brizzi et al. 2000, 2005). It is worth pointing out that the consistency of the effects on wheat germination was observed despite the difference in treatment preparation between the present study (dilution process performed using as solvent an aqueous solution of sodium bicarbonate) and the previous ones (dilution process performed using water as solvent). Moreover, in this work we studied the aging-time effect from a biological perspective, a type of approach proposed for the first time in one of our previous papers (Brizzi et al. 2011). The As 45× treatment aged for less than three months did not show a significant effect on wheat germination, whereas when aged for longer (3–12 months) the effect became significant. This can be easily observed from the cumulative trend of germinated seeds and its linear interpolation, which show strongly differentiated curves for the control (slightly decreasing over time), and for As 45× (markedly increasing over time), as previously reported (Brizzi et al. 2011). The observed reproducibility seems to suggest a specific biological effect of EDS, and confirms that the *in vitro* wheat germination model may be suitable for further studies. In fact, plant-based experiments appear suitable for basic experimentation, making it possible to overcome some of the disadvantages of clinical trials, such as placebo effects and ethical problems. Moreover, botanical bioassays rely on a very cheap and almost inexhaustible source of biological material, and they need a short time to carry out each experiment and collect a large sample of data, essential for statistical analysis (Betti et al. 2008, 2009; Majewsky et al. 2009).

The physico-chemical behavior of EDS strongly supports the significant biological effects observed in the wheat model. The measurement of specific conductivity, χ (μS cm^{-1}), was found to be extremely useful for tracking the structural alterations of the solvent water and making long-term studies possible, since the solutions are not consumed by the measurement procedures, whereas other techniques (e.g. calorimetric) are disruptive (Elia, Napoli et al. 2008). The versatility of this method allowed us to confirm previous data (Elia et al. 2004; Elia V, Elia L et al. 2007) on the physico-chemical properties of aqueous EDS: the As 45× treatment showed a highly significant increase of conductivity with respect to the solvent used to dilute (Control). In water, the high mobility of the H^+ and OH^- ions under a gradient of electrical potential is related to the presence of clusters of water molecules, held together by hydrogen bonds (the hopping mechanism) (De Grotthuss 1806; Gileadi and Kirowa-Eisner 2006). The proton hopping hypothesis, which entails an increased specific conductivity as a result of the faster ion diffusion, would account for the greater conductivity of EDS, without the need to posit an increased concentration of electrical charge carriers, i.e. of ions (Elia, Napoli et al. 2007). Based on the above cited and the present results, we hypothesize that the particular EDS preparation technique (iterated dilutions and succussions) could lead to an alteration of the structure of the solvent with the development of dissipative structures (Prigogine 1977; Montaigner et al. 2009, 2011; Marchettini et al. 2010), a variation of the supermolecular structure of the solvent water via H-bonds (Elia V, Elia L et al. 2008). These dissipative structures, through their dimensions, could augment the contribution of the hopping mechanism and hence the resultant diffusion of ions, leading to an increase of the specific conductivity (Belon et al. 2008; Cacace et al. 2009; Elia et al. 2009, 2010; Elia and Napoli 2010). The results obtained are highly self-consistent, and tend to support the working hypothesis that the hopping mechanism is augmented by the presence of molecular aggregates of water molecules. Moreover, analyzing the aging-time effect on conductivity, we observed an increase of this parameter over time. On the basis of our previous results (Elia et al. 2006; Elia V, Elia L et al. 2008; Elia, Napoli et al. 2008), we hypothesize that this conductivity increase can be attributed to two different factors: the release of alkaline oxides from glass containers, that are then converted to sodium bicarbonate in contact with the atmospheric CO_2, and the increase in concentration and/or dimension of clusters of water molecules (dissipative structures) (Elia, Napoli et al. 2007). As shown by Prigogine (1977), dissipative structures form in open systems that are far from equilibrium and in EDS they appear in the presence of a flux of matter or energy. The system under study can be considered 'open' since there is a release of alkaline oxides from glass containers (Elia V, Elia L et al. 2008); moreover, we hypothesize a flux of energy deriving from the electromagnetic background of very low frequencies (very difficult to screen), either produced by natural sources (Nickolaenko and Hayakawa 2002) or by artificial sources.

Our working hypothesis is that the increase in the germination stimulating effect could be related to an increase in the number, size or shape of the dissipative structures over time, as for conductivity. The results presented here are preliminary to a further research study and need to be repeated, as independent experiments, a number of times; in particular, in order to exclude effects from unspecific physico-chemical alterations that may occur due to the succussion procedure, diluted/succussed controls will be considered. Therefore, definite conclusions on the specific effects of EDS would be premature and the relationship observed between the increase of wheat germination and of conductivity could be of interest for further studies.

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