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Genotype differences towards lead chloride harmful action

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Abstract

The aim of the study was to throw more light on the $PbCl_2$ mode of action (MoA) depending on the genotype by the application of three model organisms and microbiological, biochemical, and molecular approaches.

Three model systems – *Chlamydomonas reinhardtii* strain 137C – wild type (WT), *Saccharomyces cerevisiae* strain D7ts1, and *Pisum sativum* L. cultivar Ran1 and two experimental schemes – short- and long-term treatments were used. *C. reinhardtii* and *S. cerevisiae* cell suspensions (1×10^6 cells/ml) at the end of the exponential and the beginning of a stationary phase of growth were treated with various $PbCl_2$ concentrations (0.45–3.6 mM) for 2 hours. Lower $PbCl_2$ concentrations (0.03–0.22 mM) were also tested on *C. reinhardtii* 137C. Short-term treatment for up to 2 days with $PbCl_2$ concentrations in the range of 0.45–3.6 mM and long-term treatment for up to 10 days with concentrations in the range of 0.45–2.7 mM was performed on *P. sativum* L. seeds and plants, respectively. Long-term treatment with a $PbCl_2$ concentration of 3.6 mM was not tested because of the very strong toxic effect (plant death). The following endpoints were used – for *C. reinhardtii*: cell survival, "visible" mutations, DNA double-strand breaks (DSBs), malondialdehyde (MDA), intracellular peroxides (H_2O_2), and photosynthetic pigments; for *S. cerevisiae* – cell survival, gene conversion, reverse mutation, mitotic crossing-over, DSBs, superoxide anions, MDA and glutathione (GSH); *P. sativum* L. – germination and root length (short-term treatment), pro-oxidative markers – MDA, H_2O_2 and photosynthetic pigments (long-term treatment).

Genotype differences between *C. reinhardtii* (0.047 mM) and *S. cerevisiae* (1.66 mM) were observed by two endpoints: concentrations inducing 50% lethality (LD_{50}) and DSB induction. By contrast, no mutagenic effect was found for both unicellular test models. A slight toxic capacity of $PbCl_2$, measured as inhibition of *Pisum sativum* L. seed germination and around 20% root length reduction was revealed after the treatment with concentrations equal to or higher than 1.8 mM.

The variety of stress responses between the two plant test models was demonstrated by comparing MDA and H_2O_2. A dose-dependent increase in H_2O_2 levels and a minor increase of MDA levels (around 9–15%) were measured when *C. reinhardtii* cells were treated with concentrations in the range of LD_{20}–LD_{80} (0.03–0.11 mM). Analyzing the kinetics of MDA and H_2O_2 in pea leaves, the most pronounced effect of concentration was shown for 2.7 mM. A decrease in the photosynthetic pigments was detected in the two experimental designs – short-term on *C. reinhardtii* and long-term on *P. sativum* treatments. The pro-oxidative potential was also proven in *S. cerevisiae* based on increased levels of MDA and superoxide anions and decreased GSH.

New information is gained that $PbCl_2$ can affect the DNA molecule and photosynthetic pigments via induction of oxidative stress. Our study revealed that the magnitude of stress response towards $PbCl_2$ is genotype-specific. Our finding that *Chlamydomonas reinhardtii* is a sensitive test system towards $PbCl_2$ contributes to good strategies for revealing very low levels of contaminants present chronically in main environmental matrices.

This is the first report, as far as we know, affirming that $PbCl_2$ can induce DSBs in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*.

Keywords

Chlamydomonas reinhardtii, DNA damaging potential, lead chloride, mutagenicity, *Pisum sativum* L., pro-oxidative effect, *Saccharomyces cerevisiae*, toxicity/genotoxicity

Introduction

Currently, environmental pollution with various chemicals is considered as an important environmental problem provoked to a large extent by human activity (Zulfiqar et al. 2019). Due to this fact, strict legislation was created, controlling both manufacturing processes, the levels released into nature as well as maximum levels of certain contaminants in food, water, air, etc. (See Commission Regulation EC documents 2016/582, 2022).

Over the years significant contamination of the main ecological matrices with heavy metals such as cadmium, lead, chromium, copper, zinc, mercury, and arsenic has been reported by different authors (Schulin et al. 2007; Kabir et al. 2012; Satta et al. 2012). The increased levels of heavy metals as a result of human activity – industry, agriculture (irrigation with contaminated water, use of mineral fertilizers), waste burning, burning of fuel, road transport, etc. has caused concern about both the health of nature and man (Järup 2003; European Commission 2013; Tóth et al. 2016).

Heavy metals commonly present in the form of cations possess two main features: quick accumulation and slow release. Mercury, cadmium, and lead are considered substantial risk factors for the biota.

Lead (Pb) is considered the second most toxic metal after arsenic (As) as it is very toxic for all living organisms (ATSDR 2019; Zulfiqar et al. 2019). Sources of lead could be dust, old paint, different user products, leaded gasoline, batteries, smelting and refining processes, etc. (Can et al. 2008; Wani et al. 2015; Kumar et al. 2020).

The poisoning capacity of lead has been known since ancient times. In the last century, toxic effects of lead and its compounds were the focus of scientists. A lot of data were collected concerning lead's high accumulation in different body tissues and organs as well as its toxic capacity as a result of exposure to contaminated water, air, and food (Balali-Mood et al. 2021; Mohanta et al. 2022).

Currently, new experimental data were gathered suggesting lead's indirect mechanisms of genotoxicity – the production of free radicals, altered expression of DNA repair genes, and inhibition of DNA repair systems (García-Lestón et al. 2010; Hemmaphan and Bordeerat 2022).

For the first time, evidence was published by Liu et al. (2018) concerning the DNA damaging potential of lead by promoting oxidative stress as well as the methylation of DNA repair genes in human lymphoblastoid TK6 cells.

Everything written above demonstrated that lead and its compounds have been characterized as toxic/genotoxic in a variety of test systems but little is currently known about their mutagenic, clastogenic, carcinogenic capacity, and mode of action (MoA).

In short, at present, information concerning the potential pro-oxidative, mutagenic, and DNA damaging potential of lead and its compounds as well as its MoA, is scarce.

Here we aimed to try to compensate for this gap to some extent using three model organisms and different approaches – microbiological, biochemical, and molecular in order to shed more light on the MoA of $PbCl_2$ depending on the genotype.

This investigation was performed using three model organisms – unicellular green alga *Chlamydomonas reinhardtii*, *Saccharomyces cerevisiae*, and *Pisum sativum* L.

Unicellular green algae, including *C. reinhardtii*, are a robust model for plant cells in genetic, molecular, physiological, and eco-toxicological studies due to their advantages which have been well described previously (Chankova et al. 2000, 2005, 2007, 2014; Chankova and Bryant 2002; Dimitrova et al. 2007). On the other hand, results obtained in this model organism could be extrapolated to a variety of plant organisms (Merchant et al. 2007; Li et al. 2020).

S. cerevisiae is an extensively used model for studying the response of heavy metals due to the highly conservative mechanisms related to different stress response pathways as well as protein similarity with higher eukaryotes including humans (Rajakumar et al. 2020; Todorova et al. 2015a). The entirely sequenced *S. cerevisiae* genome reveals around 31% similarity to the human genome and thus results could be extrapolated at human level (Todorova et al. 2015b).

Pisum sativum L. (garden pea) is a classic model organism used in biochemical, physiological, and genetic studies of plants. In addition, it should be mentioned that *Pisum sativum* L. is one of the most important food items among legume crops (Galal et al. 2021). The variety Ran 1, is a widely popular agricultural crop in Bulgaria.

Materials and methods

Model systems, cultivation, and experimental schemes

Three model systems – *Chlamydomonas reinhardtii* strain 137C – wild type (WT), *Saccharomyces cerevisiae* strain D7ts1, and *Pisum sativum* L. cultivar Ran1, and two experimental schemes – short – and long-term treatments were used. $PbCl_2$ of analytical grade was purchased from Valerus LTD.

Short-term treatment

Chlamydomonas reinhardtii 137C was cultivated at standard conditions – light of 70 μmol/m^2.s and t = 25 ± 3 °C and *Saccharomyces cerevisiae* strain D7ts1 was cultivated at t = 30 °C, 200 rpm to the end of the exponential and the beginning of a stationary phase of growth. After that, cell suspensions with a density of 1×10^6 cells/ml were treated with various $PbCl_2$ concentrations (0.45, 0.9, 1.8, 2.7, and 3.6 mM) for 2 hours. Additionally, due to the very low cell survival of *C. reinhardtii* 137C after the treatment with this concentrations' range, lower concentrations were also tested – 0.03, 0.06, 0.11, and 0.22 mM.

Pisum sativum L. seeds were treated with $PbCl_2$ at concentrations of 0.45, 0.9, 1.8, 2.7, and 3.6 mM for 24 and 48 hours to evaluate both the germination and the root length.

Long-term treatment

Pea plants were grown on a Knop medium until they reached the third physiologically developed leaf (around 10 days) under controlled conditions in a growth chamber NUVE GC 400, light regime 16/8 day/night; a temperature of 24 ± 2 °C; humidity of 70 ± 5%. After that, the Knop medium of the plants was replaced with $PbCl_2$ solutions with various concentrations in the range of 0.45, 0.9, 1.8, and 2.7 mM for 10 days. Experiments for pro-oxidative potential were conducted in order to study the kinetics of these markers. Plants were grown for 2, 5, 7, and 10 days in a medium contaminated with different $PbCl_2$ concentrations written above; leaves' samples were subsequently collected and biochemical analyses were performed.

The genotoxic potential of $PbCl_2$ on *Chlamydomonas reinhardtii* was evaluated as described in Dimitrova et al. 2014. In short, a "clonal assay" was performed to evaluate the colony-forming ability of the strain after the treatment, counting macro-colonies' survival.

On *Saccharomyces cerevisiae*, the Zimmermann test was used (Zimmermann et al. 1984).

The survival fraction (SF) (Bryant 1968) as well as concentrations that can induce 20, 50, and 80% lethality was calculated (Lidanski 1988; Dimitrova et al. 2014). The toxicity was evaluated based on the inhibition of *Pisum sativum* L. germination and reduction of root length.

Oxidative stress markers

Intracellular malondialdehyde (MDA) was measured at 532 nm and 600 nm as described by Dhindsa et al. (1981). H_2O_2 content was measured at 390 nm (Heath and Packer 1968). Pigment contents were measured at 663, 645, and 452.5 nm (Arnon 1949; Dimitrova et al. 2007) on Ultrospec 2100 pro spectrophotometer (Amersham Biosciences). Total glutathione was measured according to Zhang (2000). Superoxide anions were evaluated as described in Stamenova et al. (2008).

Mutagenic action

A test of "visible mutant colonies" was applied to evaluate the mutagenic potential of $PbCl_2$ on the unicellular algae after that the Mutagenic Index was calculated as described by (Dimitrova et al. 2007). Changes in size, morphology, and pigmentation of surviving colonies were analyzed (Shevchenko 1979).

Zimmermann's test (Zimmermann et al. 1984) with *Saccharomyces cerevisiae* diploid strain D7ts1 (*MAT*a/*a ade2-119/ade2-40 trp5-27/trp5-12 ilv1-92/ilv1-92 ts1/ts1*) was applied as described before (Todorova et al. 2015b). The test provides simultaneous detection of cell survival, mitotic gene conversion at the *trp-5* locus, and reversion mutations in the *ilv1* locus.

DNA damaging potential

DNA double-strand breaks (DSBs) were measured by Constant field gel electrophoresis (CFGE) as described in Chankova and Bryant (2002), Chankova et al. (2005), and Todorova et al. (2015a, 2019).

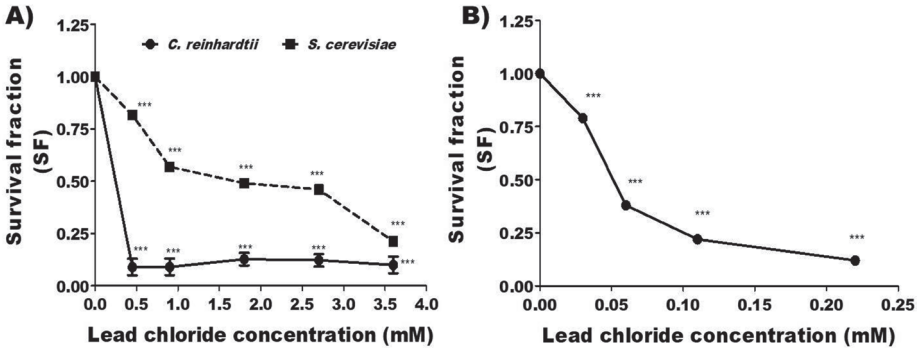
Statistical analysis

Data were analyzed using GraphPad Prism5 software (San Diego, USA) and the statistical analysis was done by one-way and two-way analysis of variances (ANOVA) followed by the Bonferroni posthoc multiple comparisons test. Linear correlation, using Pearson Product- Moment Correlation Coefficient analysis (PMCC, or r) and coefficient of determination (R^2) were determined. All the experiments were performed in triplicate. Data are presented as mean ± SEM (standard error of the mean).

Results

Genotoxicity in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*

Genotype-related differences in the response toward lead treatment were identified. Treatment with concentrations in the range of 0.45–3.6 mM $PbCl_2$ resulted



C)

	Chlamydomonas reinhardtii (WT)	*Saccharomyces cerevisiae* (D7ts1)
	Lead chloride concentration (mM)	
LD20	**0.03**	**0.47**
LD50	**0.05**	**1.66**
LD80	**0.14**	**3.67**

Figure 1. A Cell survival of *Chlamydomonas reinhardtii* (black circle) and *Saccharomyces cerevisiae* (black square) after the treatment with $PbCl_2$ concentrations in the range of 0.45–3.6 mM for 2 hours **B** cell survival of *Chlamydomonas reinhardtii* after the treatment with $PbCl_2$ concentrations in the range of 0.03–0.22 mM for 2 hours **C** three doses of lethality were calculated. Results are from at least three experiments with independently grown cell cultures and presented as mean ± SEM. Asterisks represent statistical significance (ns $P > 0.05$; *** $P < 0.001$). Where no error bars are evident, they are equal to or smaller than the values.

in a differential response of *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* (Fig. 1A). Dose-dependent decrease of cell survival ($P<0.0001$) up to 25% was obtained for *S. cerevisiae* D7ts1 (Fig. 1A). All the concentrations tested resulted in around 10% cell survival of *C. reinhardtii* 137C. No effect of the concentration was calculated.

Further, lower $PbCl_2$ concentrations were tested in *C. reinhardtii* 137C (Fig. 1B). A dose-dependent decrease of survived colonies was obtained when concentrations in the range of 0.03–0.22 mM ($P < 0.0001$) were applied ($P < 0.0001$).

Based on the survival data, three doses of lethality were calculated (Fig. 1C). Data revealed that an around 30-fold lower dose can cause 50% lethality in *C. reinhardtii* in comparison with *S. cerevisiae*.

Toxicity in *Pisum sativum* L.

A slight toxic capacity of lead chloride on *P. sativum* L. was evaluated. Around 10% inhibition of seed germination (Fig. 2A) and around 20% root length reduction (Fig. 2B) were calculated after the treatment with $PbCl_2$ concentrations equal to or higher than 1.8 mM.

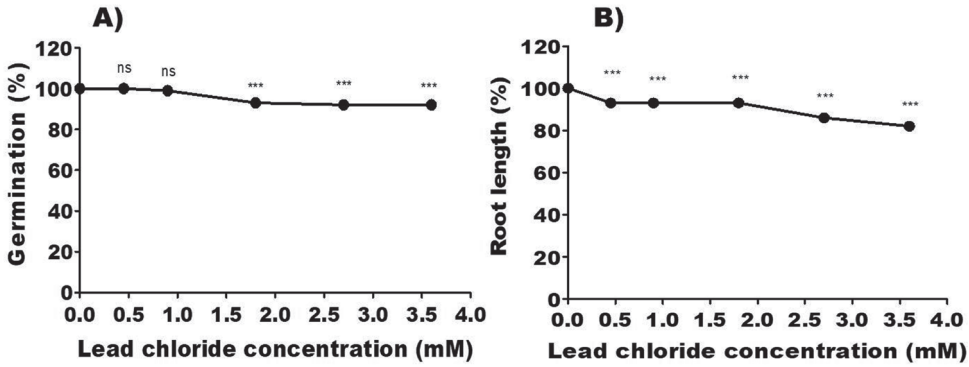


Figure 2. Toxic potential of various concentrations of $PbCl_2$ on *Pisum sativum* L. presented as percent germination (**A**) and root length (**B**). Results are from at least three experiments and presented as mean ± SEM. Asterisks represent statistical significance (ns $P > 0.05$; *** $P < 0.001$). Where no error bars are evident, they are equal to, or smaller than, the values.

Even though the decreases in both parameters were calculated as statistically significant to the corresponding control sample, they can hardly be regarded as significant from a biological point of view.

Pro-oxidative potential after short-term treatment with $PbCl_2$ on the unicellular model systems – *C. reinhardtii* and *S. cerevisiae*

The pro-oxidative potential of $PbCl_2$ was studied by several endpoints in the range of concentrations corresponding to LD_{20}, $LD_{50,}$ and LD_{80}.

A very minor increase in MDA levels (around 9–15%) (Fig. 3) was measured when *C. reinhardtii* cells were treated with concentrations in the range of LD_{20}–LD_{80} (0.03–0.11 mM) ($P < 0.001$).

Concerning the other pro-oxidative marker, dose-dependent statistically significant higher levels of intracellular peroxides were obtained (Fig. 3).

More than 3-fold higher levels of both MDA and H_2O_2 were induced after the treatment with concentrations that can cause over 80% cell lethality.

As a next step, the levels of photosynthetic pigments were evaluated (Fig. 3). A similar – around 50–60% decrease in chlorophyll *a* (chl *a*), chlorophyll *b*, and carotenoids were calculated without concentration dependence ($P < 0.0001$).

Concerning the other model system – *S. cerevisiae*, treatment with concentrations corresponding to LD_{20}, LD_{50}, and LD_{80} resulted in a statistically significant dose-dependent increase in the superoxide anions' levels (Table 1). The most pronounced effect – more than 2-fold higher levels of superoxide anions was calculated at LD_{80} ($P < 0.01$). Around a 2-fold increase with no effect of the concentration was obtained for MDA (Table 1). At the same time, a decrease in the total glutathione levels was measured for the same experimental conditions ($P < 0.0001$).

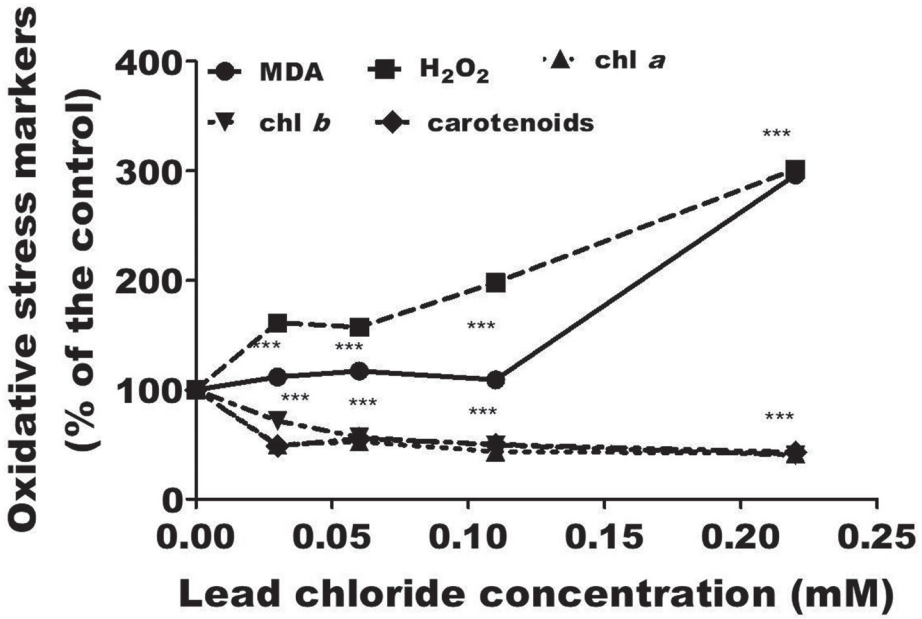


Figure 3. Oxidative stress induced by $PbCl_2$ at concentrations range of 0.03–0.22 mM in *Chlamydomonas reinhardtii*. Data are presented as mean ± SEM. All the results are from at least three independent experiments. Statistical significance was calculated among all the samples and to the controls ($P < 0.001$). Where no error bars are evident, they are equal to or smaller than the values.

Table 1. Oxidative stress markers in *Saccharomyces cerevisiae* after the treatment with various $PbCl_2$ concentrations for 2 hours.

	Superoxide anions (pM O2-/cell)	MDA (mM/g sample)	GSH (mmol GSH/g sample)
Control	0.404 ± 0.01	0.128 ± 0.039	0.003 ± 0.00006
LD_{20}	0.360 ± 0.04ns	0.260 ± 0.027*	0.002 ±0.00006***
LD_{50}	0.626 ± 0.03*	0.280 ± 0.012*	0.001 ±0.00006***
LD_{80}	0.965 ± 0.05***	0.296 ± 0.009**	0.001 ±0.00007***

Mean values were calculated from at least 3- experiments by independently grown cell cultures. Asterisks represent statistical significance between the control and the samples (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Pro-oxidative potential after long-term treatment with $PbCl_2$ on *P. sativum* L.

No statistically significant differences were measured in the levels of MDA, intracellular peroxides, and the photosynthetic pigments (chl *a*, chl *b*, chl *a*/*b*, and carotenoids) in control samples grown without $PbCl_2$ for 2, 5, 7 and 10 days (data not shown).

The calculation of kinetics data shows that plants grown in an environment contaminated with different $PbCl_2$ concentrations for 2 days did not suffer at these experimental conditions. No statistically significant increase in MDA and intracellular peroxides was defined (data not shown).

Table 2. Kinetics of the oxidative stress markers MDA and intracellular H_2O_2 in *Pisum sativum* L. after long-term treatment (2, 5, 7, and 10 days) with various $PbCl_2$ concentrations.[1]

Concentrations	Marker	Days		
		5	7	10
Control	MDA	100	100	100
	H_2O_2	100	100	100
0.45 mM	MDA	140±10.87***	133±2.86***	125±2.66**
	H_2O_2	99±3.47 ns	96±9.80 ns	113±1.55 ns
0.9 mM	MDA	131±6.34***	123±1.63*	111±3.70 ns
	H_2O_2	93±2.55 ns	91±2.46 ns	100±3.32 ns
1.8 mM	MDA	121±7.64*	123±3.86*	127±4.77**
	H_2O_2	97±3.49 ns	104±10.02ns	128±8.65**
2.7 mM	MDA	129±9.88**	143±5.54***	166±7.53***
	H_2O_2	119±3.12 ns	129±9.85**	172±9.43***

[1] Results are calculated as a percent of the control. Data are presented as mean ± SEM. All the results are from at least three independent experiments. Asterisks represent statistical significance between the control and the treated samples (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Treatment with $PbCl_2$ concentrations from 0.45 to 1.8 mM resulted in an approximately similar increase in both MDA and H_2O_2 levels (Table 2). No effect of exposure time was found. The most pronounced oxidative stress measured as increased MDA and H_2O_2 contents was calculated when plants were grown for 10 days in a contaminated with 2.7 mM $PbCl_2$ medium.

A similar relationship was defined concerning the other marker for oxidative stress induced by $PbCl_2$ and measured as levels of H_2O_2. The only statistically significant increase was calculated when plants were treated for 10 days with concentrations of 1.8 and 2.7 mM (Table 2).

Based on these results, it could be speculated that in our experimental conditions, $PbCl_2$ most probably induces lipid peroxidation in *Pisum sativum* L. plants.

Here, we have not discussed the effects obtained after the applications of the most favorable for *Pisum sativum* L. plants experimental conditions – the lowest concentration of 0.45 mM $PbCl_2$ and two days duration of plants growing at different $PbCl_2$ concentrations due to the fact that both factors have not affected in any way photosynthetic pigments contents (data not shown).

Concerning the other concentrations in the range of 0.9–2.7 mM, data revealed that the photosynthetic pigments are affected mostly by the concentration and not by the treatment time (Fig. 4A–C). The only time-dependent decrease (Fig. 4B) was detected for chl *b* and treatment with 0.9 mM ($P < 0.001$).

Our results have revealed the harmful potential of $PbCl_2$ on *P. sativum* L. plants grown for 2, 5, 7, and 10 days in a medium contaminated with various $PbCl_2$ concentrations. We found a statistically significant decrease in the levels of photosynthetic pigments we analyzed by around 40–60%, as well as no changes in the chl a/b ratio compared with those in the controls samples.

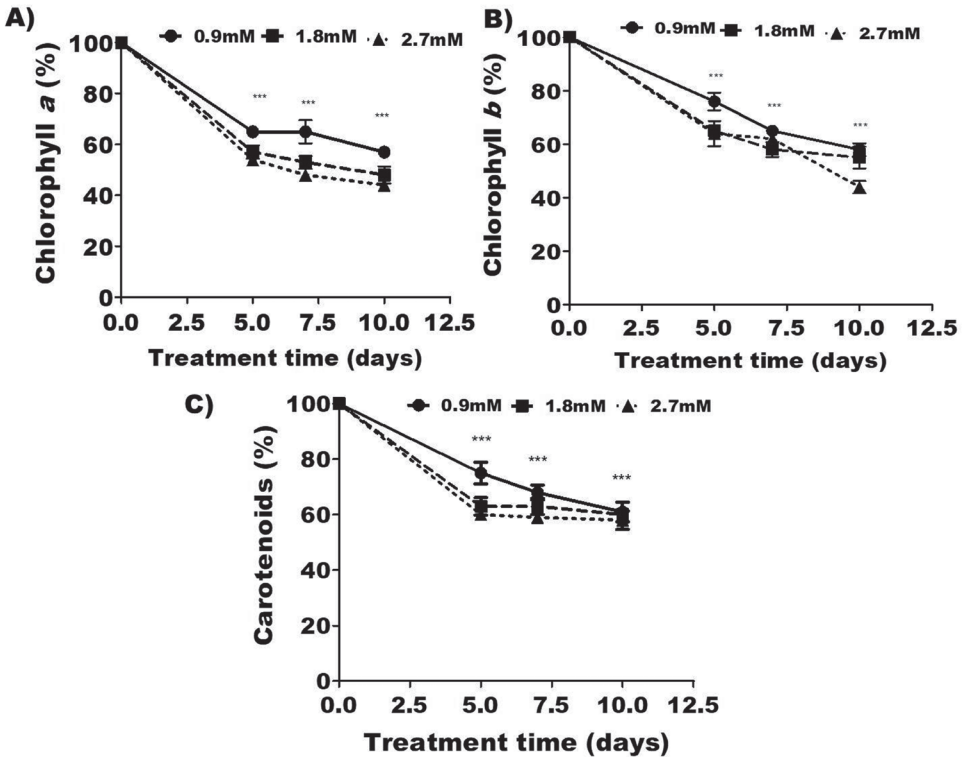


Figure 4. Kinetics of the photosynthetic pigments chlorophyll *a* (**A**), chlorophyll *b* (**B**), and carotenoids (**C**) in *Pisum sativum* L. treated with various $PbCl_2$ concentrations for different time (2, 5, 7, and 10 days). Results are calculated as a percent of the control. Data are presented as mean ± SEM from at least three independent experiments. Asterisks represent statistical significance between the control and the treated samples (*** $P < 0.001$). Where no error bars are evident, they are equal to, or smaller than. the values.

Our further steps were to clarify whether $PbCl_2$ would have some mutagenic and DNA damaging capacity on both unicellular test systems – *Chlamydomonas reinhardtii* strain 137C and *Saccharomyces cerevisiae* strain D7ts1 at our experimental conditions.

Mutagenic activity

No mutagenic potential of $PbCl_2$ was revealed on both unicellular organisms, despite the well-pronounced genotoxic effect. The mutagenic index in *Chlamydomonas reinhardtii* was calculated to be less than 2.5, indicating no mutagenic capacity of $PbCl_2$ in the tested concentration range. The mitotic gene conversion and reverse mutations in *Saccharomyces cerevisiae* were comparable with the control untreated cells (data not shown).

DNA damaging activity

To throw more light on the mode of action (MoA) of $PbCl_2$, its potential to induce double-strand breaks in DNA was evaluated on *C. reinhardtii* (Fig. 5A, C) and

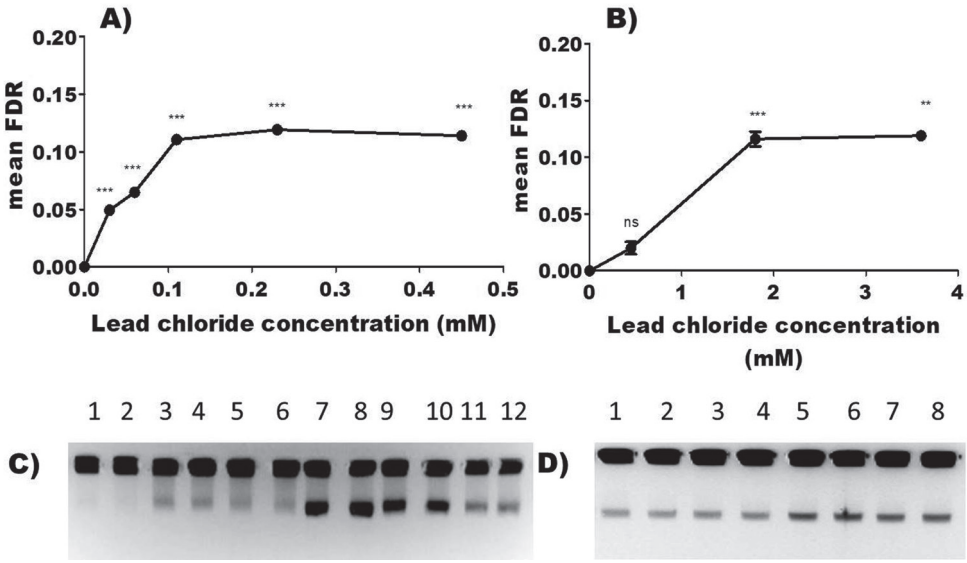


Figure 5. Levels of primary induced double-strand breaks in DNA of *Chlamydomonas reinhardtii* (**A, C** where 1,2 – control; 3, 4 – 0.03 mM; 5, 6 – 0.06 mM; 7, 8 – 0.11 mM; 9, 10 – 0.22 mM; 11, 12 – 0.45 mM) and *Saccharomyces cerevisiae* (**B, D** where 1, 2 – control; 3, 4 – 0.5 mM; 5, 6 – 1.7 mM; 7, 8 – 3.7 mM) after the treatment with various $PbCl_2$ concentrations. Values represent the mean fraction of DNA released (FDR). Data are presented as mean ± SEM. All the results are from at least three independent experiments (ns $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$). Where no error bars are evident, they are equal to, or smaller than, the values.

S. cerevisiae (Fig. 5B, D). Our data is the first experimental evidence for the induction of DSBs after the treatment with $PbCl_2$. Again, genotype-related differences in the response to $PbCl_2$ were established.

A statistically significant dose-dependent DSBs increase was found after the application of $PbCl_2$ in concentrations up to LD_{80} – 0.11 mM for *C. reinhardtii* (Fig. 5A, C). No statistically significant difference was calculated between the effect of the treatment with 0.11 and 0.22 mM $PbCl_2$ – around 2-fold elevated DSBs levels. The treatment with a $PbCl_2$ concentration of 0.45 mM resulted in significant DNA degradation (Fig. 5C).

Interesting results were obtained when comparing the DSB induced by concentrations corresponding to the calculated LD. Concentrations corresponding to LD_{80} in both test systems resulted in a similar induction of DSBs – around 2-fold (Fig. 5A, B). Around 1.5-fold higher DSB levels in *C. reinhardtii* and more than 2-fold in *S. cerevisiae* were measured after the treatment with the respective LD_{50} concentrations. LD_{20} resulted in a small but statistically significant (P<0.001) increase in DSB levels for *C. reinhardtii* (Fig. 5A) and no effect on *S. cerevisiae* (Fig. 5B).

Comparing the concentration ranges, it should be pointed out that concentrations used in *Saccharomyces cerevisiae* experiments were approximately 10-fold higher than those used for *Chlamydomonas reinhardtii*.

In order to reveal whether a relationship exists among the pro-oxidative, DNA damaging, toxic/genotoxic, and mutagenic potential of lead chloride, correlation analysis was performed for both unicellular model systems.

Correlation analysis

In *Chlamydomonas reinhardtii* (Table 3), the decrease in cell survival (SF) was found to correspond strongly with an increase in the H_2O_2 ($P < 0.001$) and DSB ($P < 0.001$). A good correlation was calculated between higher levels of H_2O_2 and low levels of all the photosynthetic pigments (Table 3).

Based on this and the graphically presented changes in the markers studied (Fig. 6A), it could be speculated that the Pb-induced oxidative stress in terms of intracellular peroxides probably may participate in the induction of DSBs, resulting in a decrease in the cell survival.

Concerning the other unicellular organism – *Saccharomyces cerevisiae*, a statistically significant strong correlation was calculated between the decrease in cell survival and the induction of superoxide anions (R = -963, $P < 0.05$) (Fig. 6B). The increase in DSBs levels (Fig. 6C) was found to strongly correlate to the increase in MDA levels (R = 0.947, $P < 0.05$) and the decrease in GSH (R = -0.964, $P < 0.05$). The changes in the markers could be observed in Fig. 6B, C.

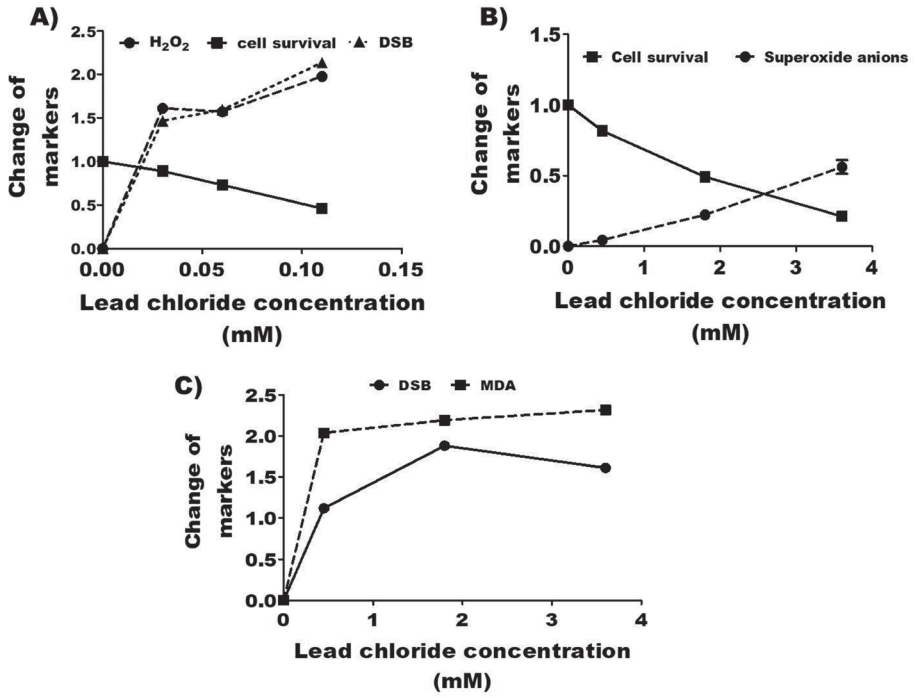


Figure 6. Marker's correlation in *C. reinhardtii* (**A**) and *S. cerevisiae* (**B**) after 2 hours treatment with PbCl2 in concentrations corresponding to LD_{20}, LD_{50} and LD_{80}.

Table 3. Correlation analysis among the studied endpoints in a model system *Chlamydomonas reinhardtii* after the treatment with $PbCl_2$.

	SF	DSB	MDA	H_2O_2	chl *a*	chl *b*	Car
SF		-0.942**	-0.717	-0.943**	0.763	0.944**	0.721
DSB			0.587	0.941**	-0.916*	-0.979**	-0.863
MDA				0.825	-0.561	-0.653	-0.646
H_2O_2					-0.888*	-0.953**	-0.889*
chl *a*						0.907*	0.985**
chl *b*							0.871
Car							

[1] Studied endpoints in the range of $PbCl_2$ concentrations 0.03–0.22 mM – MDA, H_2O_2, chl *a*, chl *b*, carotenoids (car), survival fraction (SF), and double-strand breaks (DSB). Values represent the R^2 for linear correlation. A correlation coefficient (R) higher than 0.900 denotes a strong positive correlation and higher than -0.900 – a strong negative correlation (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Discussion

Lead is a very toxic non-trace metal with well-proven both poisonous and genotoxic capacities (Oztetik 2021; Riyazuddin et al. 2022; Chmielowska-Bąk et al. 2022). Recently, it was reported that the bioactivity of lead and its compounds can be attributed to its pro-oxidative capacity (Devóz et al. 2021; Chmielowska-Bąk et al. 2022). As stated in the introduction, information concerning the potential pro-oxidative, mutagenic, and DNA-damaging potential of lead and its compounds as well as its MoA is scarce.

Our main aim was to supply new information about the MoA of $PbCl_2$. This aim has provoked us to focus our attention on two main items: the first relates to the evaluation of mutagenic and DNA-damaging capacity of $PbCl_2$ on two model test systems; the second was to analyze the possible contribution of oxidative stress in these biological events using various endpoints.

According to the WHO, a battery of test systems and endpoints evaluating different adverse effects at different levels is a good strategy for obtaining reliable information about MoA of different xenobiotics. Additionally, the application of several test systems provides more reliable information as some tested materials, such as chelating agents, heavy metals, and some surfactants with unusual physical and chemical properties, may cause practical and test system-specific difficulties, and compromise the outcome of the test by providing false-negative or -positive results (Turkez et al. 2017).

The advantages of the test systems were described briefly in the introduction. They were chosen based on the fact that each of them may provide information concerning different endpoints. Such a strategy may provide more detailed information concerning the MoA of xenobiotics. For the purpose of our study, this investigation has gone through several consecutive steps, using a complex of approaches – microbiological, biochemical, and molecular.

The first one was to evaluate the toxic/genotoxic capacity of tests-systems and the results obtained were compared with some experimental data of other authors.

The toxic capacity of $PbCl_2$ on *P. sativum* L. cultivar Ran1 was evaluated by two endpoints – inhibition of seed germination and reduction of root length. It can be said

that the minor toxic effect of $PbCl_2$ in our experimental scheme is not concentration-dependent, in spite of the fact that differences were statistically significant. Our observation confirms the one reported by Silva et al. (2017) that growth-related parameters could not be considered as the most sensitive and reliable endpoints to evaluate Pb toxicity. The slight toxicity of $PbCl_2$ on *Pisum sativum* L. presented as slightly inhibited germination and reduced root length have been reported by other authors and other plants such as *Parkinsonia aculeata* and *Pennisetum americanum* (Shaukat et al. 1999); *Ipomoea aquatica* Forsk (Ni'am and Yuniati 2021) and *Hordeum vulgare* L.) (Vasić et al. 2020). Ni'am and Yuniati (2021) speculate that the stronger effect on root length than germination could be due to the low permeability of seed testa to lead.

Based on the toxic/genotoxic results, additional data were provided concerning the sensitivity of *C. reinhardtii* strain 137C to $PbCl_2$ compared with *S. cerevisiae*. The high sensitivity of *C. reinhardtii* was previously reported for chlorpyrifos (Todorova et al. 2020). This finding is important because very sensitive genotypes are a very good tool for revealing very low levels of contaminants present chronically in main environmental matrices, and can result in long-term disturbance of biota.

Further, the pro-oxidative potential of $PbCl_2$ was confirmed in all the model systems used by us. The approach applied by us on the test systems covers a wide range of reactive oxygen species (ROS). It is well-known that the first ROS produced is the superoxide anion (Sullivan and Chandel 2014). A dose-dependent increase was calculated for the levels of superoxide anions in *S. cerevisiae* D7ts1, which is in accordance with such findings in *S. cerevisiae* haploid strains (Dimitrov et al. 2011; Sousa and Soares 2014) and *Pisum sativum* L. root cells (Malecka et al. 2001). It is well-known that the $O_2^{\cdot -}$ is easily converted to H_2O_2 by the enzyme mitochondrial superoxide dismutase (Sod2) (Herrero et al. 2008; Sousa and Soares 2014). Thus, we can assume that probably part of the superoxide anions could be converted to peroxides. As a next step, the levels of intracellular peroxides were studied. Data revealed that *P. sativum* L. and *C. reinhardtii* responded differently to $PbCl_2$-induced oxidative stress in terms of intracellular peroxides. While no effect was observed for most of the concentrations tested on *P. sativum* L., a dose-dependent increase was obtained for *C. reinhardtii*. It can be suggested that the genotype also plays a role in the response to the $PbCl_2$-induced oxidative stress.

The next marker for oxidative stress studied was MDA. Interestingly, the response significantly varies depending on the genotype. Our study provides evidence that although an increase in the MDA levels has been observed for all the model systems, the sensitivity of the marker depends on the genotype and the experimental conditions. In the unicellular organisms, MDA was not affected in a dose-dependent way, suggesting that it may not be the primary consequence of $PbCl_2$ treatment for 2 hours. Oppositely, in our long-term experiments on *P. sativum* L., the levels of MDA were found to be the most increased compared to the rest of the pro-oxidative stress markers. Contradictory data exist concerning the induction of lipid peroxidation by lead and its compounds. According to some authors, lead may play an indirect role in lipid peroxidation (Sivaprasad et al. 2004; Hasanein et al. 2017). Based on results in the

present work, it may be speculated that $PbCl_2$-induced lipid peroxidation depends on the experimental conditions.

The last marker studied was GSH. Its role in the antioxidant defense against various stressors is well documented (Perez et al. 2013). The results presented in this work also confirm those published by Perez et al. (2013) that $PbCl_2$ reduces the levels of GSH. The authors suggest that such a reduction could be explained by the high affinity of the GSH thiol group to Pb and thus the formation of Pb-GSH complexes in the cytosol, decreasing the level of GSH (Perez et al. 2013).

In addition to the well-known poisonous capacity of lead and lead compounds, our results confirmed and extended the current state of knowledge regarding their indirect mechanism of genotoxicity via induced oxidative stress. The specificity of induced radicals was found to depend on genotype and experimental conditions.

Further, the effect of $PbCl_2$ on the photosynthetic machinery was evaluated. Interestingly, all the photosynthetic pigments in *Chlamydomonas reinhardtii* and *Pisum sativum* L. decreased in a similar way by around 40–50% in both experimental schemes – short-term and long-term treatment without concentrations or time-dependence.

Contradictory data exist concerning the effect of lead on photosynthetic pigments. Some studies point out that lead treatment may result in a decrease in the chlorophyll content of *Phaseolus vulgaris* and *Lens culinaris* which may be attributed to the ability of lead to replace the magnesium (Mg) in the chlorophyll ring (Irfan et al. 2010; Rai et al. 2016) as well as in other plants (Ruley et al. 2006; Yang et al. 2015). Other authors have reported that Pb does not affect the photosynthetic pigments in *Scenedesmus acutus*, *Schroederia* sp. (Dong et al. 2022), and *Pisum sativum* (Rodriguez et al. 2015).

According to Zheng et al. (2020), the reduction of cell growth of *Chlamydomonas reinhardtii* could be due to the reduction of the chlorophyll content. The Pb-induced disturbance of photosynthesis in microalgae has been proposed as a major cause of cell death by Li et al. (2021). In our study, no statistically significant correlation was obtained between these markers. The SF was found to be negatively affected by H_2O_2 which confirms other findings that the metal-induced oxidative stress in *Chlamydomonas reinhardtii* is related to growth inhibition (Bertrand and Poirier 2005; Stoiber et al. 2013). It should be taken into account that the increase in H_2O_2 also correlates to the decrease in photosynthetic pigments.

Here we can speculate that $PbCl_2$ can affect DNA molecules and photosynthetic pigments via the induction of oxidative stress.

In the present work, an attempt was made to evaluate the potential mutagenic and DNA damaging potential of $PbCl_2$. No mutagenic effect was obtained for both unicellular organisms – *C. reinhardtii* and *S. cerevisiae* at our experimental schemes. Our results confirm those published by Francisco et al. (2018) that Pb does not possess a mutagenic effect on *Allium cepa* root meristematic cells. In the present work, two tests for mutagenic activity were applied each of them providing information concerning various types of genetic alternations. Our data provide indirect evidence that $PbCl_2$ toxicity at the tested concentrations may not be related to the induction of point mutations, impaired cell division; micro-chromosomal aberrations; altered cell

wall structure and composition, mitotic gene conversion and mitotic crossing-over (Zimmermann et al. 1984; Dimitrova et al. 2007).

To the best of our knowledge, the present work provides the first evidence that $PbCl_2$ induces DSBs in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*. It could be speculated that the Pb-induced oxidative stress could be the major mechanism for the induction of double-strand breaks in DNA which, in turn, may partially result in cell death.

Conclusion

Our study revealed that the magnitude of stress response towards $PbCl_2$ is genotype-specific. *Chlamydomonas reinhardtii* 137C is a more sensitive to $PbCl_2$ model than *Saccharomyces cerevisiae* D7ts1 and *Pisum sativum* L. cultivar Ran1. The approach applied by us provided additional information concerning the mode of action of $PbCl_2$. The toxic/genotoxic and DNA-damaging potential of $PbCl_2$ may be a result of the pro-oxidative effect. This is the first report, as far as we are aware, that lead chloride may induce DSB in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*. Our proposed approach – a battery of test systems and various endpoints could be considered a promising tool in the ecotoxicological assessment of various xenobiotics.

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