

The effects of CaCl₂ and CaBr₂ on the reproduction of *Daphnia magna* Straus

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Concentrated CaCl₂ and CaBr₂ salt solutions of densities up to 2.3 kg L⁻¹ are regularly used to control hydrostatic pressure in oil wells during special operations in the exploration and production of natural gas and crude oil. Various concentrations of high density salts are frequently left in mud pits near the drilling site as waste, polluting fresh and ground waters by spillage and drainage. The toxic effects of these salts have already been observed. This study investigated the effects of CaCl₂ and CaBr₂ on water flea *Daphnia magna* Straus in a 21-day reproduction test. The three tested concentrations of CaCl₂ (240, 481, and 1925 mg L⁻¹) caused a significant dose-response decrease of reproduction ($p < 0.001$). With CaBr₂ (533 and 1066 mg L⁻¹), only aborted eggs were produced, demonstrating the embryotoxicity of the substance. The results suggest that high concentrations of the tested chemicals are harmful to *Daphnia*'s reproduction and could reduce its abundance.

KEY WORDS: acute immobilisation test; bromide embryotoxicity; chronic reproduction test; high density salts

Concentrated solutions of calcium chloride (CaCl₂) and calcium bromide (CaBr₂), densities from 1.39 to 2.30 kg L⁻¹, and their mixtures commonly known as high density salts or clear brines, are extensively used as completion or workover fluids in oil and natural gas exploration and production industry. Their main function is to provide the required density that controls subsurface pressure in the formations with abnormally high pressure, as well as to minimize formation damage, maintain borehole stability, transport moveable solids, and suspend solids, all of which maximise the recovery of hydrocarbons from the production reservoir (1-3). After operations, high density brines are often left in the mud pits near the well site as waste and may pollute fresh and groundwater by spilling over and draining through the ground.

Several studies investigating the toxic effects of concentrated solutions of CaCl₂ and CaBr₂ have been performed using plant test systems and an animal test organism, the freshwater snail *Planorbarius corneus* (Linnaeus, 1758) (4-8). Studies on plants (4-7) showed a significant inhibition of duckweed (*Lemna minor*) growth at higher concentrations of CaCl₂ (5550, 8325, and 9624 mg L⁻¹) and CaBr₂ (14931 and 21320 mg L⁻¹). Photosynthetic pigments chlorophyll *a* and *b* increased and the stress indicator anthocyanin were also increased in greater duckweed (*Spirodella polyrrhiza*) (4-7). Growth inhibition was observed in the green algae *Chlorella kessleri*

at 21320 mg L⁻¹ CaBr₂ (5). The study on *P. corneus* showed a significant dose response decrease of survival and fecundity (egg-mass number) in chronic toxicity test at concentrations ranging from 1203 to 4813 mg L⁻¹ CaCl₂, 1066 to 5329 mg L⁻¹ CaBr₂, and 774 to 5801 mg L⁻¹ of their mixture 1:1 (8).

To further explore the toxic effects of CaCl₂ and CaBr₂, observed in our previous study on *P. corneus* (8), we selected *Daphnia magna* Straus as a model system to perform a semistatic 21-day reproduction test. This species frequently occupies habitats in north-western Croatia, where gas and oil exploration and production are performed. Furthermore, water fleas as primary grazers and primary forage for invertebrates and vertebrates represent ecologically important constituents of food webs and are also an established model commonly used in toxicology (9, 10).

MATERIALS AND METHODS

Daphnia laboratory culture

Daphnids originating from a local wild population and an unpolluted area were reared in the laboratory culture in 1000 mL glass vessels at a density of 50 individuals per L in dechlorinated tap water passed through activated charcoal. They were fed daily with a concentrated suspension of fresh cultured green algae *Chlorella vulgaris* and *Scenedesmus obliquus* alternately with an addition of suspended dry baking yeast once a week and kept at

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20 ± 2 °C at 12/12 photoperiod (550 lux). Once a week, daphnids were transferred to fresh water and neonates were regularly removed.

Acute and chronic semi-static toxicity test procedure

Concentrations of the chemicals for chronic reproduction test were chosen based on 24 and 48 h preliminary exposure. Acute and chronic tests were conducted according to the OECD standard procedures (11, 12). For the acute exposure, four replicates for each treatment and control were used with five neonates for each replicate. There were five concentrations for both tested chemicals. Animals were kept under the same laboratory conditions as those in the chronic test but were not fed. Acute exposure to CaCl_2 showed 100 % mobility after 48 h in 240 and 481 mg L^{-1} and 60 % mobility in 1925 mg L^{-1} and for CaBr_2 90 and 80 % mobility for 533 and 1066 mg L^{-1} respectively, so the chronic reproduction test was set with the same chemical concentrations.

For the chronic experiments, neonates from 3rd-5th brood ≤ 24 h old (from mothers previously acclimated in dilution water) were placed individually in 50 mL of test solution in glass beakers covered with watch glass. Ten replicates per test solution and ten replicates as a control were kept at room temperature (20 ± 2 °C), at 12:12 photoperiod. Animals were fed daily with 4×10^5 cell mL^{-1} of the algae species *Chlorella vulgaris* and *Scenedesmus obliquus* 2:1. Three times a week the animals were transferred to a fresh test solution and test beakers were positioned in a random manner within the testing area, neonates and aborted eggs were recorded and discarded, survival was recorded daily. Dilution water used for preparing test solutions and the controls (ISO test water) contained 11.76 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.93 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.59 g NaHCO_3 , and 0.23 g KCl.

Each salt was diluted in deionised water to 1 L and 25 mL of each solution was added to prepare 1 L of dilution water. The dilution water had a conductivity of $< 10 \mu\text{S cm}^{-1}$, pH 7.8 and hardness 250 mg L^{-1} CaCO_3 (11). ISO test water contains 294 mg L^{-1} CaCl_2 so each exposure CaCl_2 concentration was an addition to that content. All chemicals used in the experiments were produced by Merck (Darmstadt, Germany) and of p.a. grade, except CaBr_2 which was extra pure grade. The actual concentrations of the chemicals in the test solutions were measured once a week after preparing fresh solutions and they varied from the nominal concentrations ± 0.1 to 5 %. Calcium was measured with a Varian Techron AA5 atomic absorption spectrophotometer (13), bromide was measured by the spectrophotometric method (14), and chloride was measured by silver nitrate volumetric method (15).

Statistical analysis

The experiments were statistically analysed with one-factorial ANOVA and Tukey *post hoc* test to reveal the statistical differences between treatments (16), using Statistica 6 (StatSoft, Tulsa, AZ, USA). The half maximal effective concentration EC_{50} values were calculated using regression analysis and Bonferroni test and the significance of females' mortality was determined using Cochran-Armitage trend test (12).

RESULTS

The results are given in Table 1 and Figures 1 and 2. Our results of the acute immobilisation tests showed that the EC_{50} 48-hour value for CaCl_2 was 2020 mg L^{-1} ($p=0.01$) and for CaBr_2 2238.6 mg L^{-1} ($p=0.003$).

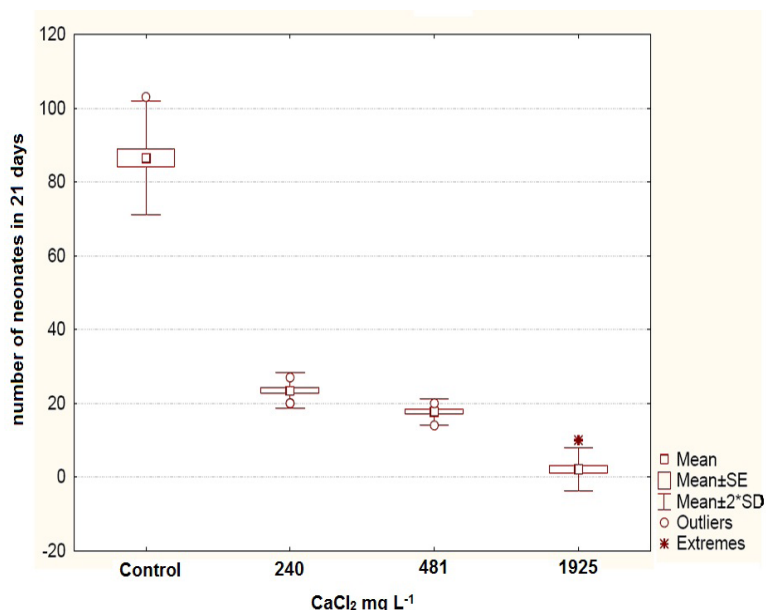


Figure 1 Effect of CaCl_2 on reproduction of *Daphnia magna*: mean number of living neonates per female (number of female at the start of the test which did not inadvertently or accidentally die during the test, determined by Cochran-Armitage trend test)

Table 1 Reproduction and survival of *Daphnia magna* exposed to CaCl₂ and CaBr₂ for 21 days

Group	No. of neonates	No. of broods	Brood size	Days to 1 st brood	Viable neonates/ surviving female/day	No. of aborted eggs	Females' mortality (n)	Longevity (Days)
Control	85.5±7.72	4.5±0.4	20.7±3.31	9.7±0.34	4.1±0.34	0	1	20.9±0.32
CaCl ₂ 240 mg L ⁻¹	23.4±2.41*	4.1±0.3	5.7±0.53*	9.8±1.03	1.1±0.12*	0	2	20.8±0.42
CaCl ₂ 481 mg L ⁻¹	17.7±1.77*	4.3±0.4	4.8±0.81*	10.2±0.79	0.8±0.08*	0	3	20.6±0.70
CaCl ₂ 1925 mg L ⁻¹	2.1±3.00 [#]	0.9±0.7 [#]	1.6±3.02 [#]	11.0±1.15	0.26±0.14 [#]	0	8	16.5±3.84
CaBr ₂ 533 mg L ⁻¹	-	3.5±1.74	5.4±2.54*	10.2±0.53	-	21.9±14.2	6	-
CaBr ₂ 1066 mg L ⁻¹	-	2.7±1.62	3.2±1.80*	12.5±5.41 [§]	-	10.9±7.22	5	-

The number of females for each group was 10

Results are expressed as mean±SD

SD=standard deviation

*Significant difference between groups and the control (p<0.001)

[#]Significant difference compared to other groups (p<0.001)

[§]Significant difference compared to other groups (p<0.01)

Statistical differences between groups were determined using one-factorial ANOVA and Tukey post hoc test

The results of chronic reproduction tests showed a significant dose-related reduction of neonate production in all test solutions with CaCl₂ compared to the control (p<0.001). At 240 and 481 mg L⁻¹ CaCl₂, the neonate production was significantly higher than at 1925 mg L⁻¹ (p<0.001) and mortality was lower by 20 and 30 %, respectively. The greatest reduction appeared with the highest concentration of 1925 mg L⁻¹ CaCl₂ which caused 80 % mortality (Figure 1, Table 1). Parental mortality in exposed replicates followed a concentration–response pattern as determined by Cochran-Armitage trend test. The EC₅₀ value for reproduction was 900 mg L⁻¹.

Both concentrations of CaBr₂ were highly toxic to adults (50 and 60 % mortality) and embryos (100 %) so only aborted eggs were found. There were twice as many produced eggs in the lower concentration of CaBr₂ than in the higher concentration (Figure 2, Table 1).

As shown in Table 1, the first-brood day was significantly delayed only in 1066 mg L⁻¹ CaBr₂. The average number of neonates per brood decreased in all three CaCl₂ concentrations and the average number of broods significantly decreased only in the most toxic solution of CaCl₂, 1925 mg L⁻¹ (Table 1).

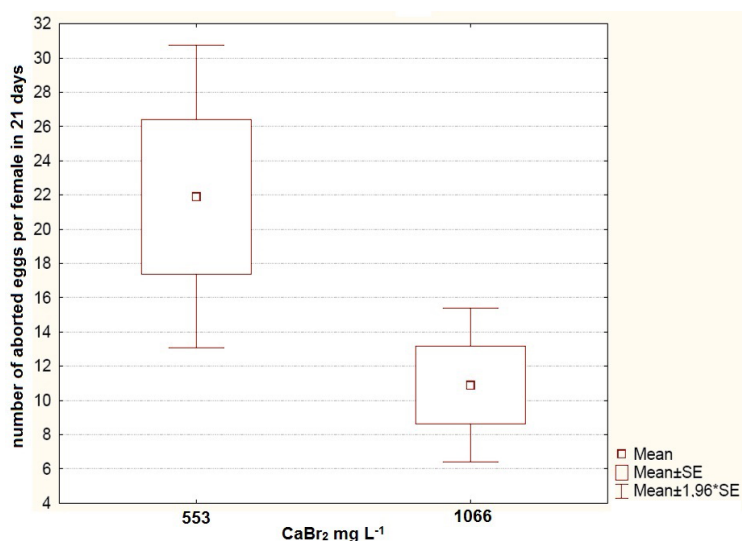


Figure 2 Effect of CaBr₂ on reproduction of *Daphnia magna*: mean number of aborted eggs per female

DISCUSSION

A research study on the toxicity of CaCl₂ reported that the EC₅₀-48 h for *Daphnia magna* was 2190 mg L⁻¹ (17). The EC₅₀-48 h value of 2020 mg L⁻¹ determined in this study is very similar to the reported value for the acute toxicity of CaCl₂. The results of the reproduction test on daphnids in sublethal concentrations of CaCl₂ from the same study (17) showed a significantly lower reproduction compared to the control at the concentration of 1173 mg L⁻¹, but not at lower concentrations (235 and 496 mg L⁻¹). The highest CaCl₂ concentration investigated in our present study (1925 mg L⁻¹ CaCl₂, corresponding to 1230 mg L⁻¹ Cl⁻) caused 80 % mortality and a significant loss of reproduction in the 21-day reproduction test. The two lower concentrations (240 and 481 mg L⁻¹) were also significantly toxic so the calculated EC₅₀ amounted to 900 mg L⁻¹. The difference between the reported results and ours could be attributed to the different sensitivity between the *D. magna* clones or culture conditions (18, 19).

A report on the toxicity of chloride to high sensitive cladocerans (20) stated that the chronic toxicity threshold value for reproduction inhibition is 421 mg L⁻¹ Cl⁻ (*Daphnia magna* 21-day IC₂₅) and 454 mg L⁻¹ Cl⁻ (*Ceratodaphnia dubia* 7-day IC₂₅); toxicity tests measuring chloride toxicity were based on using NaCl to minimize the toxicity contributed by the more toxic counter cation such as K⁺, Mg²⁺, Ca²⁺. The lowest concentration investigated here (240 mg L⁻¹ CaCl₂, corresponding to 153.3 mg L⁻¹ Cl⁻) that reduced neonate production to 27 % of the control indicated that CaCl₂ could be more toxic than NaCl.

There is a lack of information on the chronic toxicity of CaBr₂ to *Daphnia magna* but data on chronic and acute toxicity of NaBr are widely distributed in the literature. The NOEC (No Observed Effect Concentration) on fecundity generally ranges from <3 to >117 mg L⁻¹ NaBr (19). The differences in results may depend on the clone chosen to run the test and laboratory environment (18, 19, 21). Some of the reported acute toxic values are: LC₅₀ of 7900 and EC₅₀ for reproduction of 29 mg L⁻¹ NaBr (22), EC₅₀ of 5700 to 10800 mg L⁻¹ NaBr (23), EC₅₀ 24 h of 37.37 mmol L⁻¹ NaBr (24), etc. Hermens et al. (25) reported an LC₅₀ 48 h of 13500 and an EC₅₀ (16-day reproduction) of 29 mg L⁻¹ NaBr and concluded that the effect on reproduction for compounds like NaBr is at much lower levels than for acute mortality. Sloof and Canton (26) reported that NaBr strongly affected daphnid reproduction in a semi-chronic test. Their NOLC (No Observed Lethal Concentration) was 3200 mg L⁻¹ and NOEC (reproduction) 10 mg L⁻¹ NaBr. Based on the results of the long term toxicity tests on NaBr, Canton et al. (27) found that bromide had a marked effect on the reproduction of fresh water organisms (*D. magna*, *Poecilia reticulata*, and *Lymnaea* sp., NOEC was 7.8 mg L⁻¹ Br⁻) - the bromide ion was proven to have an embryotoxic effect. Investigating chronic bromide stress on *D. magna* reproduction in chronic toxicity experiments, Leeuwen et

al. (28) found that bromide did not delay the onset of reproduction nor the brood frequency, but disturbed embryonic development so that at 100 mg L⁻¹ NaBr reproduction was completely inhibited and only aborted eggs were found, but survival was unaffected at up to 10000 mg L⁻¹. Therefore, the authors concluded that bromide inhibited the reproduction process.

The results of the present study on CaBr₂ are in good agreement with the former as there were only aborted eggs released by females, significant delay to the onset of reproduction was observed only at 1066 mg L⁻¹ CaBr₂ but not at 533 mg L⁻¹. The mean brood frequency in CaBr₂ was lowered to 3.5 and 2.7 due to the high mortality of the females before the end of the test. Our results indicate that CaBr₂ could be more toxic than NaBr, as it caused 50 and 60 % mortality at much lower concentrations than reported for NaBr (26, 28); however, these differences could also be explained with clonal and culturing differences (18, 19, 21). Many toxicants can cause partial or complete abortion of clutches of eggs and embryo abnormalities e.g. NaBr and 3,4-dichloroaniline (29), ibuprofen (30), and insecticides (31). An increased number of aborted eggs and a reduction in the number of hatched individuals have been documented in NaCl solutions at concentrations of Na ranging from 25 to 249 mg L⁻¹ Na⁺ and 41 to 249 mg L⁻¹ Na⁺, respectively, in chronic exposure of the freshwater cladoceran *Pseudosida ramosa* (32, 33). Baird et al. (29) reported that the chronic inhibition of reproduction in *D. magna* at 3 to 17 mg L⁻¹ NaBr was due solely to the acute lethal effect on eggs developing in the brood chamber. The decreased number of aborted eggs in higher concentration of CaBr₂ compared to the lower one in the present study could indicate that such a high concentration of bromide (1066 mg L⁻¹ CaBr₂, 852 mg L⁻¹ Br⁻) could also affect the production of eggs in the ovarium and not only development in the brood chamber.

Comparing the results of this investigation with those from our previous study on the freshwater snail *Planorbarius corneus*, it can be concluded that *Daphnia magna* is more sensitive to the toxic effect of the chemicals than *Planorbarius corneus*.

The results of this study have also shown that high concentrations of CaCl₂ are toxic to *Daphnia magna* reproduction, reducing offspring production. CaBr₂ showed an embryotoxic effect preventing neonate production. The contamination of freshwater with the investigated chemicals near drilling sites could have a harmful effect on *Daphnia magna* reproduction and reduce the population by reducing its abundance.

Taken together and generally speaking, we can conclude that our findings suggest the need for a more detailed clarification of the toxicity profiles of CaCl₂ and CaBr₂, primarily because they have demonstrated significant negative impacts on daphnid reproduction. Future studies should focus on examining the effects of these two compounds on other freshwater species.

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Učinak CaCl₂ i CaBr₂ na razmnožavanje vodenbuhe (*Daphnia magna* Straus)

Koncentrirane otopine soli CaCl₂ i CaBr₂ gustoće do 2,3 kg L⁻¹ redovito se koriste za kontrolu hidrostatskoga tlaka u bušotinama tijekom posebnih operacija u istraživanju i proizvodnji prirodnoga plina i sirove nafte. Različite koncentracije soli visoke gustoće često su bile ostavljene u isplačnim jamama u blizini područja bušenja te su prelijevajući se ili ocjeđujući u podzemlje zagađivale slatke i podzemne vode. Iako je dosad već provedeno nekoliko istraživanja toksičnoga učinka koncentriranih otopina soli CaCl₂ i CaBr₂, u ovom radu istraživana je učinka CaCl₂ i CaBr₂ na vodenbuhu *Daphnia magna* u dvadesetjednodnevnom testu reprodukcije. Tri ispitane koncentracije CaCl₂ (1925, 481 i 240 mg L⁻¹) prouzročile su značajan, o dozi ovisan pad reprodukcije ($p < 0,001$). U otopinama CaBr₂ (533 i 1066 mg L⁻¹) proizvedena su samo abortirana jaja, što pokazuje embriotoksičnost te tvari. Rezultati upućuju na to da su visoke koncentracije ispitivanih tvari štetne za razmnožavanje vodenbuhe te mogu smanjiti brojnost tih organizama.

KLJUČNE RIJEČI: akutni test imobilizacije; embriotoksičnost bromida; kronični test reprodukcije; soli visoke gustoće