Phyton (Horn, Austria)	**Vol. 45**	Fasc. 2	183–196	30. 12. 2005

Effects of $CaCl_2$ and $CaBr_2$ on Growth, Photosynthetic Pigments and Ion Accumulation in Duckweed

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With 7 Figures

Received July 28, 2005

Key words: Calcium chloride, calcium bromide, high density brines, photosynthetic pigments, duckweed, *Lemna minor*.

Summary

VIDAKOVIĆ-CIFREK Ž., WONISCH A., TAUSZ M. & GRILL D. 2005. Effects of $CaCl_2$ and $CaBr_2$ on growth, photosynthetic pigments and ion accumulation in duckweed. – Phyton (Horn, Austria) 45 (2): 183 – 196, with 7 figures. – English with German summary.

Duckweed (*Lemna minor* L.) was exposed to solutions of calcium chloride, calcium bromide and their 1:1 mixture, commonly used as oil industry high density brines. These solutions were added into nutrient media in volumes appropriate to achieve the following final concentrations: 0.025, 0.05, 0.075 and 0.1 mol L^{-1}. The two higher concentrations (0.075 and 0.1 mol L^{-1}) of all three tested samples caused disturbed water content in plants after 14 days of cultivation. On media supplemented with $CaCl_2$, certain accumulation of chloride, depending on the concentration of tested salts, was detected. After the treatment with $CaBr_2$, chloride content was lower in comparison to control. Mixture (1:1) of the both chemicals caused slightly increased chloride content, except when was present in concentration 0.1 mol L^{-1}. $CaBr_2$ and 1:1 mixture in concentrations 0.025, 0.05 and 0.075 mol L^{-1} showed evi-

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dent increase of bromide content. Comparing the calcium content in control and treated plants, a significant difference has not been established. Generally, chlorophyll *a* and chlorophyll *b* content was increased only after treatment with lower concentrations (0.025 and 0.05 mol L^{-1}) of samples that contained bromine. The main reason for high density brines effect on duckweed observed in the present investigations as well as in previous studies could be the chloride and bromide accumulation in plants as well as increased osmotic effects of two higher concentrations applied.

Zusammenfassung

VIDAKOVIĆ-CIFREK Ž., WONISCH A., TAUSZ M. & GRILL D. 2005. Auswirkungen von $CaCl_2$ und $CaBr_2$ auf das Wachstum, die Pigmente und den Ionenhaushalt von *Lemna minor* L. – Phyton (Horn, Austria) 45 (2): 183 – 196, 7 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Gesättigte Salzlösungen von $CaCl_2$ und $CaBr_2$, sowie unterschiedliche Mischungsverhältnisse der beiden Lösungen, sind bevorzugte Bestandteile von Spülflüssigkeiten bei Bohrungen zur Erdgas und Rohölgewinnung. Im unmittelbaren Bereich solcher Bohrungsstätten besteht die Gefahr, dass pflanzliche Organismen erhöhten Salzkonzentrationen ausgesetzt sind. Um die Auswirkungen dieser Salzlösungen in Hinblick auf deren potentielle Akkumulation in den Pflanzen zu untersuchen, wurden Pflanzen der Kleinen Wasserlinse (*Lemna minor* L.) mit $CaCl_2$- und $CaBr_2$-Lösungen, sowie mit einer 1:1 Mischung aus beiden Lösungen, behandelt. Die Lösungen wurden einer Nährlösung beigemengt, um ein jeweiliges Endvolumen von 0.025, 0.05, 0.075 und 0.1 mol L^{-1} zu erlangen. Nach einer 14-tägigen Behandlung wurden die Pflanzen der jeweiligen Ansätze in Hinblick auf eine Akkumulierung von Chlorid und Bromid anhand einer HPLC-Methode untersucht. Die Behandlung mit $CaBr_2$ führte zu geringeren Konzentrationen von Chlorid im Vergleich zu den Kontrollansätzen. Die Mischlösung (1:1) verursachte leicht erhöhte Chloridkonzentrationen, mit Ausnahme der höchst konzentrierten Lösung (0.1 mol L^{-1}). $CaBr_2$-Behandlungen und 1:1 – Mischlösungen der Konzentrationen 0.025, 0.05 und 0.075 mol L^{-1} zeigten deutlich erhöhte Bromidkonzentrationen. Hinsichtlich der Kalziumgehalte konnten, im Vergleich zur Kontrolle, keine erhöhten Konzentrationen festgestellt werden. Die Analysen der Pigmentgehalte, die durchgeführt wurden, um potentielle Stresseinflüsse der Salze aufzuzeigen, ließen keine Rückschlüsse auf ein erhöhtes Stressaufkommen in den Pflanzen zu.

Introduction

Saturated water solutions of calcium chloride, calcium bromide (densities 1.30 kg L^{-1} and 1.61 kg L^{-1}, respectively) and their mixtures in different proportions are commonly used as high density brines for pressure control in oil wells during exploration and production of natural gas and crude oil (SCHMIDT & al. 1983). Accidental spills of these solutions could pollute nearby fresh and ground waters and soil. Animal and plant organisms living close to oil wells could become exposed to increased concentration of calcium, chloride and bromide which are main constituents of high density brines.

The physiological and cytogenetic effects of high density brines have been investigated in freshwater snail *Planorbarius corneus* L. (MAŽURAN & al. 1999) green alga *Chlorella kessleri* Fott & Novák (VIDAKOVIĆ-CIFREK & al. 1999), shallot *Allium cepa* L. var. *ascalonicum* (VIDAKOVIĆ-CIFREK & al. 2002) but mostly in duckweed (*Lemna minor* L.).

Duckweed *Lemna minor* L. is a small floating freshwater monocotyledon widely used for evaluation of the physiological effects of environmental pollutants due to its small size, rapid growth, vegetative reproduction, ease of culture and high sensitivity to numerous chemicals (WANG 1986, LEWIS 1995). Therefore it has been chosen for previously done and present studies of physiological effects of high density brines.

VUJEVIĆ & al. 2000 found out that 0.025 mol L^{-1} of $CaCl_2$, $CaBr_2$ and their 1:1 mixture had stimulative effect on duckweed growth rate. Concentration 0.050 mol L^{-1} did not affect the growth significantly, while two higher concentrations (0.075 and 0.1 mol L^{-1}) reduced it.

There were no significant differences between the three investigated solutions of high density brines when they were prepared in the same concentrations. Chlorophylls and carotenoids as indicators of exposure to stress (LICHTENTHALER 1987) were evaluated spectrophotometricaly after two weeks of exposure and increased contents (expressed on fresh weight basis) of chlorophyll *a* and *b* were noticed (TKALEC & al. 1998, VUJEVIĆ & al. 2000). Changed chloroplast morphology and sedimentation profile in sucrose gradient caused by the two higher concentrations of high density brines (0.075 and 0.1 mol L^{-1}) could be the consequence of starch accumulation (VIDAKOVIĆ-CIFREK & al. 2001) which leads to increased proportion of dry weight (VUJEVIĆ & al. 2000). VUJEVI? & al. 2000 compared also the effect of high density brines of technical grade (commonly used in oil industry) with the effect of $CaCl_2$ and $CaBr_2$ pure chemicals and noticed that the chemicals of different degree of purity had very similar effect on duckweed growth. Therefore, the obtained effect was due to the main constituents of high density brines (Ca^{2+}, Cl^- and Br^-) and not of inorganic impurities.

All influences of high density brines on *Lemna minor* described so far could be the consequences of increased osmotic value of nutrient solution supplemented with high density brines, as well as by accumulation of the main constituents of these solutions (calcium, chloride and bromide) in plant tissue.

In the present study growth parameters as well as calcium, chloride and bromide content in duckweed tissue cultivated on media containing high density brines was measured to find out if the effect of high density brines was due to increased osmotic value only or accumulation of ions also contributed to observed effects.

Since accumulation of various elements can influence photosynthetic pigments, determination of chlorophyll *a* and *b* content as indicators of plant response to stress was also included in investigations.

Material and Methods

Tested Samples

Saturated water solutions of $CaCl_2$ and $CaBr_2$ (contained 481.3 g L^{-1} and 1065.9 g L^{-1}, respectively) of technical grade, commonly used as high density brines, have been used as tested samples. These solutions, as well as their 1:1 mixture were added into the modified Hoagland's nutrient solution in volumes appropriate to achieve the following concentrations: 0.025, 0.05, 0.075 and 0.1 mol L^{-1}. 1:1 mixture samples (1 part of $CaCl_2$: 1 part of $CaBr_2$) contained the both of the tested solutions in such volumes that both salts together achieved the total concentrations of 0.025, 0.05, 0.075 or 0.1 mol L^{-1}.

Atomic absorption spectrophotometry and volumetric method were used to determine an accurate amount of calcium, chloride, and bromide and other inorganic substances in tested samples (Tkalec & al. 1998). Besides these main constituents of high density brines, the analysis showed certain amounts of magnesium and zinc, but after dilution of tested samples with nutrient medium these impurities, due to their low concentration, cannot have influence on tested plants, as confirmed in our previous study in which we compared the chemicals of analytical and technical grade (Vujević & al. 2000). Amounts of heavy metals (Cd, Cr, Ni, V, Fe and Co) were under detectable levels. Detection limits for those metals were (mg L^{-1}): Cd = 0.0005, Cr = 0.07, Ni = 0.008, V = 0.1, Fe = 0.005 and Co = 0.006.

Plant Material and Culture Conditions

Stock cultures of duckweed, *Lemna minor* L. were maintained under axenic conditions on the modified Pirson-Seidel's nutrient solution, pH 4.55 (Pirson & Seidel 1950, Krajnčič 1974) and subcultured biweekly.

All cultures, stock and experimental, were grown in controlled chamber conditions under 16 hours of light (80 μmol m^{-2} s^{-1}) at 24 $\pm$ 2 °C.

Experimental Cultures

Two experimental series were run: the first for growth determination and second for calcium, chloride and bromide as well as chlorophyll content determination. In the both experiments duckweed was exposed to tested solutions for two weeks.

Growth Determination

For growth determination the experiment was carried out in 100 ml Erlenmeyer flasks containing 60 ml of modified Hoagland nutrient solution, pH 5.0 (Krajnčič & Devidé 1980) supplemented with tested samples in concentrations mentioned above. Control plants were grown under the same conditions without addition of tested samples into nutrient solution. The healthy colony with 2-3 fronds was transferred into each Erlenmeyer flask. All treatments and control was prepared in eight replicates. The frond number and fresh weight were determined on the last day of the experiment and results were expressed as fresh weight to frond number ratio.

Dry to fresh weight ratio was determined in plants used for ion analysis.

Ion Analysis and Chlorophyll Content

Experimental and control cultures were established by inoculation of 7–8 healthy colonies from stock cultures into 300 ml Erlenmeyer flasks containing 150 ml of modified Hoagland's nutrient solution. After 14 days of cultivation plants were washed three times with deionised water.

For ion analysis plants were gently dried with paper towel and oven-dried at 105 °C for 48 h and powdered in a dismembrator (Braun Mikro-Dismembrator II, Braun, Maria Enzersdorf, Austria).

Before and after oven-drying procedure fresh and dry weight of plants was determined, respectively.

Calcium was analysed in 200–300 mg of dried, powdered, samples digested in mixture of concentrated nitric acid (2.5 ml) and sulphuric acid (6 ml) using Büchi (Switzerland) apparatus. Calcium content was determined by atomic absorption spectrophotometry (Shimadzu AA-660) and results (mg Ca g^{-1}DW) expressed as mean values of three independent experiments.

Chloride and bromide contents were determinated by Schöninger combustion followed by an isocratic HPLC-method. HPLC-system consisted of a LDC Milton Roy CM 400 pump, a Metrosep Anion Dual 1 column (3 × 150 mm), and a conductivity detector (ESA IonChem Model 5400). Solvent was 8 mmol L^{-1} phthalic acid / 2 % acetonitrile / pH 4.0 (TRIS). Flow rate was 0.5 ml min^{-1}.

Bromide is not a common constituent of plant tissue therefore its content was determined only in plants cultivated on media supplemented with tested solutions containing bromide.

Obtained data (mg g^{-1}DW) represent mean value of five independent experiments.

For photosynthetic pigments' content plants were frozen in liquid nitrogen, lyophilized and stored in darkness at –20 °C until analysis. The lyophilized plants were powdered in a dismembrator. The dry powder (50 mg) was extracted two times in acetone (1 ml, 1 min on a Vortex mixer) and after centrifugation (17500 g at +4 °C) the both supernatants were brought to 3 ml with acetone. Acetone extracts (20 µl) were injected into HPLC using a cooled (+4 °C) autosampler.

Pigments in acetone extracts were determined according to the HPLC gradient method described by Pfeifhofer 1989. Column: Sperisorb S5 ODS2 250 × 4.6 mm with pre-column S5 ODS2 50 × 4.6 mm; Solvent A: acetonitrile : methanol : water = 100 : 10 : 5 (v/v/v); solvent B: acetone : ethylacetate = 2 : 1 (v/v); linear gradient from 10% solvent B to 70% solvent B in 18 min; run time 30 min; flow = 1 ml min^{-1}; photometric detection at 440 nm.

Obtained data are expressed as mg g^{-1}DW and represent mean value of three independent experiments.

Statistical Analysis

Due to the small sample size a non-parametric analysis of variance followed by Newman-Keul's Test was applied (Zar 1996).

Results

Growth Parameters

The growth expressed as fresh weight per number of fronds was inhibited after two weeks of cultivation on medium supplemented with $CaCl_2$, $CaBr_2$ or their 1:1 mixture in concentrations 0.075 and 0.1 mol L^{-1} as well as by $CaCl_2$ in concentration 0.05 mol L^{-1} (Fig. 1).

Dry weight portion was significantly increased by 0.075 mol L^{-1} $CaCl_2$ and 1:1 mixture and by all three treatments in concentration 0.1 mol L^{-1} (Fig. 2).

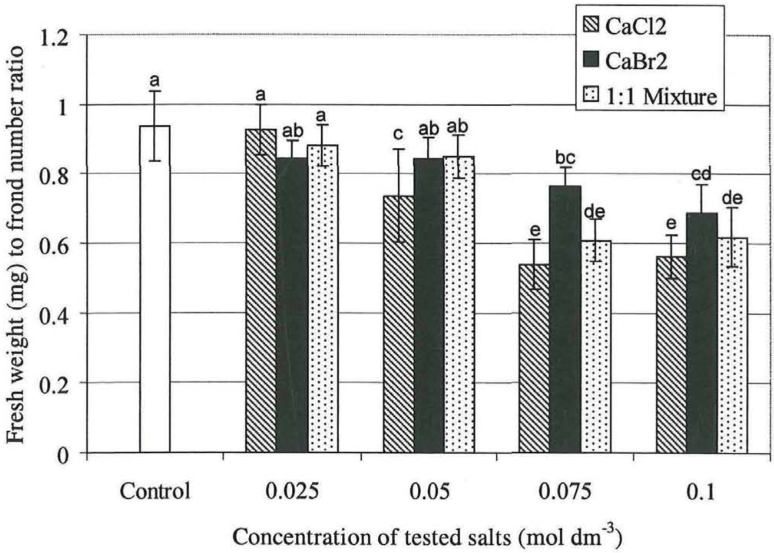

Fig. 1. Fresh weight to frond number ratio in duckweed *Lemna minor* L. after two weeks exposure to nutrient solutions supplemented with $CaCl_2$, $CaBr_2$ and their 1:1 mixture. Each value is the mean of eight replicates with standard deviation. Different letters on the top of the columns indicate significant differences between treatments at $P < 0.05$ by Newman-Keuls Test.

Calcium Content

Comparing the calcium content in control and treated plants, significant difference has not been established. However, there were some differences between treatments. The treatment with the 0.025 mol L^{-1} $CaBr_2$, 0.075 mol L^{-1} $CaCl_2$ and mixture as well as with all three treatments in concentration 0.1 mol L^{-1}, caused significantly lower calcium accumulation in comparison to treatment with 0.025 mol L^{-1} $CaCl_2$ (Fig. 3).

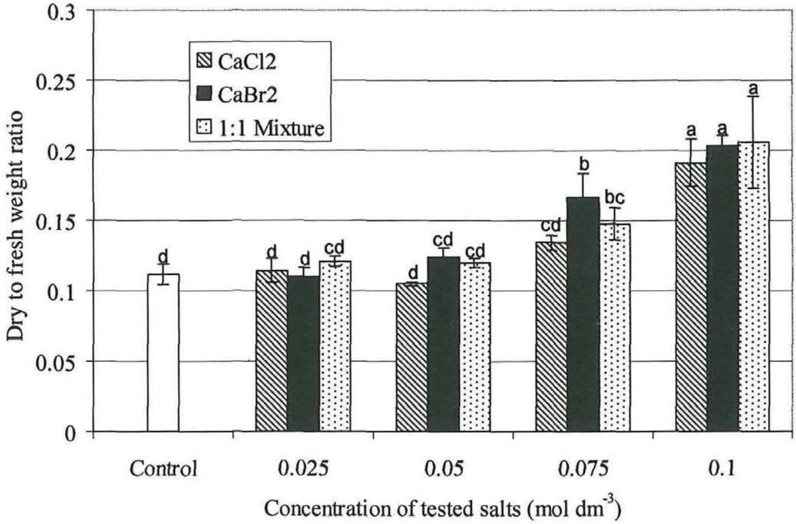

Fig. 2. Dry to fresh weight ratio in duckweed *Lemna minor* L. after two weeks exposure to nutrient solutions supplemented with $CaCl_2$, $CaBr_2$ and their 1:1 mixture. The values are the mean of three independent experiments. Different letters on the top of the columns indicate significant differences between treatments at $P < 0.05$ by Newman-Keuls Test.

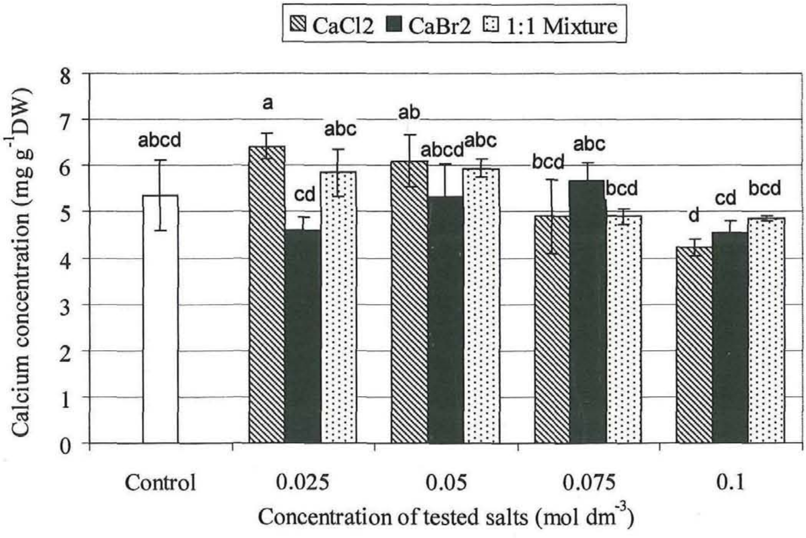

Fig. 3. Calcium content in duckweed *Lemna minor* L. after two weeks exposure to nutrient solutions supplemented with $CaCl_2$, $CaBr_2$ and their 1:1 mixture. The values are the mean of three independent experiments with standard deviation. Different letters on the top of the columns indicate significant differences between treatments at $P < 0.05$ by Newman-Keuls Test.

Chloride and Bromide Content

All four investigated concentrations of $CaCl_2$ caused increased chloride content in comparison with the control. The most prominent effects revealed treatments with concentrations of 0.05 and 0.075 mol L^{-1}. After the treatment with $CaBr_2$, chloride content was lower in comparison with the control. Mixture (1:1) of the both chemicals caused slightly increased chloride content, except in the concentration 0.1 mol L^{-1} (Fig. 4).

Plants treated with $CaBr_2$ and 1:1 mixture in concentrations of 0.025 and 0.05, as well as with 0.075 mol L^{-1} of $CaBr_2$ accumulated between 37 and 47 mg g^{-1}DW bromide. Mixture in concentration 0.075 mol L^{-1} as well as $CaBr_2$ and mixture in concentration 0.1 mol L^{-1} showed significantly lower accumulation of bromide - between 4.5 and 13.7 mg g^{-1}DW (Fig. 5).

Photosynthetic Pigments' Content

Pigment analysis of duckweed plants' extracts by HPLC separated chlorophyll *a*, chlorophyll *b*, lutein, violaxanthin, neoxanthin, antheraxanthin and β-carotene.

Compared to control, content of chlorophyll *a* was significantly increased after treatment with 0.025 mol L^{-1} and 0.05 mol L^{-1} of $CaBr_2$ as

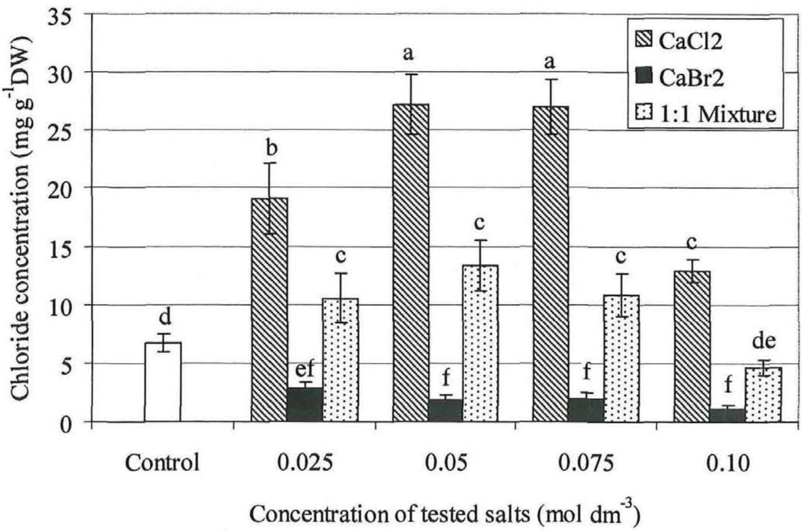

Fig. 4. Chloride content in duckweed *Lemna minor* L. cultivated for two weeks on nutrient solutions supplemented with $CaCl_2$, $CaBr_2$ and their 1:1 mixture in concentrations 0.025, 0.05, 0.075 and 0.1 mol L^{-1}. The values are the mean of five independent experiments with standard deviation. Different letters on the top of the columns indicate significant differences between treatments at $P < 0.05$ by Newman-Keuls Test.

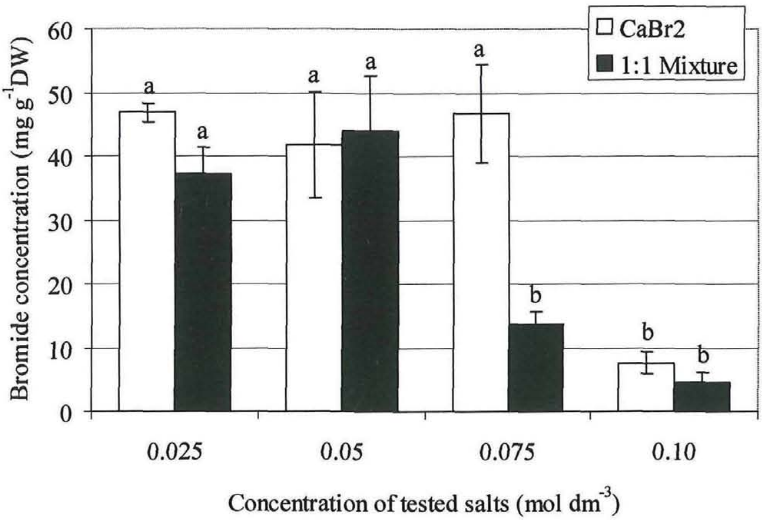

Fig. 5. Bromide content in duckweed *Lemna minor* L. cultivated on nutrient solutions supplemented with $CaBr_2$ and 1:1 mixture of $CaCl_2$ and $CaBr_2$ in concentrations 0.025, 0.05, 0.075 and 0.1 mol L^{-1}. The values are the mean of five independent experiments with standard deviation. Different letters on the top of the columns indicate significant differences between treatments at $P < 0.05$ by Newman-Keuls Test.

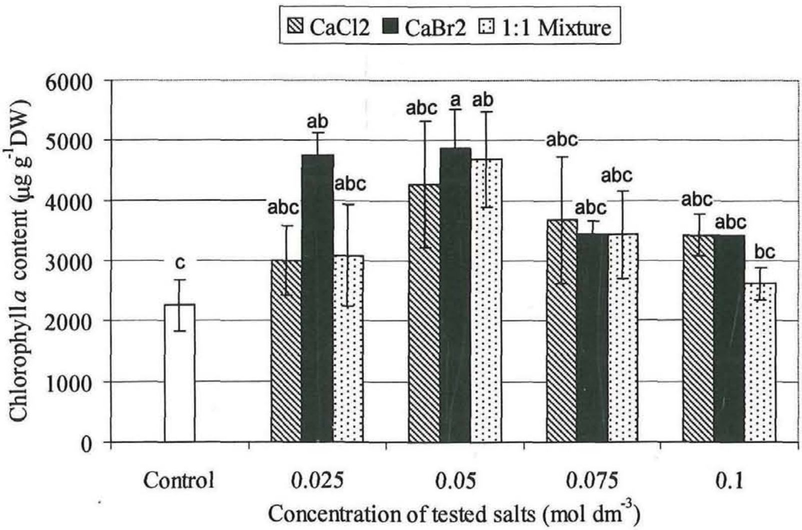

Fig. 6. Chlorophyll *a* content in duckweed plants treated with oil industry high density brines. The values are the mean of three independent experiments with standard deviation. Different letters on the top of the columns indicate significant differences between treatments at $P < 0.05$ by Newman-Keuls Test.

well as with 0.05 mol L^{-1} of mixture, while chlorophyll *b* was significantly increased by 0.025 mol L^{-1} $CaBr_2$ and 0.05 mol L^{-1} of mixture (Figs. 6 and 7). Generally, only lower concentrations (0.025 and 0.05 mol L^{-1}) of tested solutions containing bromide caused increased chlorophyll content. Chlorophyll *a/b* ratio, xanthophylls and β-carotene were not affected by applied treatments (data not shown).

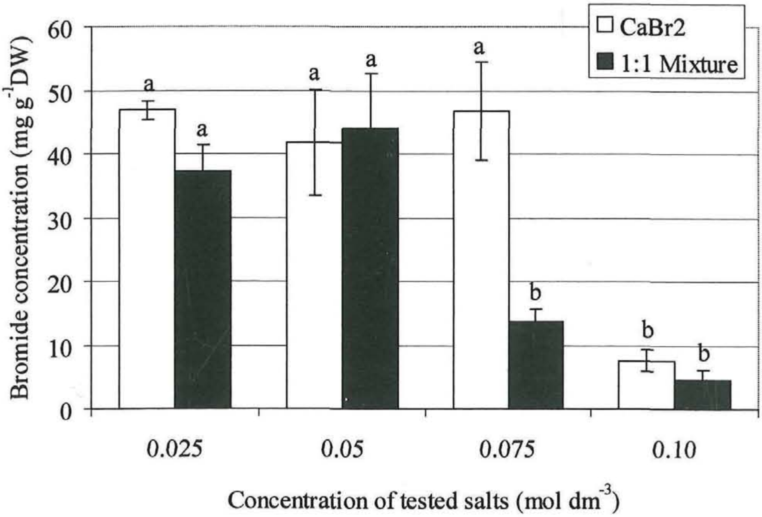

Fig. 7. Chlorophyll *b* content in duckweed plants treated with oil industry high density brines. The values are the mean of three independent experiments with standard deviation. Different letters on the top of the columns indicate significant differences between treatments at $P < 0.05$ by Newman-Keuls Test.

Discussion

According to hypothesis of OERTLI 1975 growth can be stimulated in a slightly saline environment as a result of an increased turgor, which is due to intensified ion absorption (von SURRY & FLÜCKIGER 1983). Although VUJEVIĆ & al. 2000 observed stimulated multiplication rate on media containing 0.025 mol L^{-1} of tested salts, in our experiment neither the concentration 0.025 mol L^{-1}, nor the 0.05 mol L^{-1} increased fresh weight expressed as fresh weight per frond number (Fig. 1). Therefore these treatments did not show the effect on water accumulation as a consequence of ion absorption and observed stimulative effects on plants multiplication could be due to better growth conditions established by addition of low amounts of salts (LUCKEY & al. 1975). Considering results obtained by measurement of dry weight portion (Fig. 2), significant increase after treatment with concentrations of 0.025 and 0.05 mol L^{-1} has also not been noticed so probably the established osmotic value was small enough to be overcome.

On the contrary, the addition of higher concentrations of tested salts into nutrient solution caused salt stress that could disturb uptake, translocation and distribution of nutrient elements (LECHNO & al. 1997) and lead to decreased growth as already been observed by TKALEC & al. 1998 and VUJEVIĆ & al. 2000. In presented experiment the treatment with higher concentrations (0.075 and 0.1 mol L^{-1}) of all three tested samples caused the decrease of fresh weight per frond (Fig. 1) as well as an increased portion of dry weight (Fig. 2). It could be the consequence of water loss under influence of high osmotic value of nutrient media rather than on the synthesis of biomass. Decreased growth of duckweed due to osmotic effect caused by 0.1 mol L^{-1} of mannitol confirmed TKALEC & al. 2001.

It is well-known that salinity inhibits plants growth not only by lowering the water potential in the medium, than also by alteration in the plant ionic status (GREENWAY & MUNNS 1980). Our results demonstrate that in comparison to control, calcium content was not increased in plants cultivated in the media containing $CaCl_2$ (Fig. 3). Plants have to keep free cytosolic calcium concentration at optimal level, so the excess calcium has to be transported into vacuoles (MARMÉ 1985, cit. in FRANCESCHI 1989, FRANCESCHI 1989) or possibly into the apoplast. In our experiment plants were cultivated on Hoagland's nutrient solution containing about 5 mmol L^{-1} calcium, so they have already accumulated certain amount of calcium. Therefore, the plants' capacity for calcium deposition could be overcome and accumulation of additional amount of calcium was not significant.

Contrary to calcium, chloride levels were raised after all treatments containing only $CaCl_2$, as well as $CaCl_2$ in combination with $CaBr_2$ as 1:1 mixture (Fig. 4). Chloride in plant cell has a role in osmoregulation, charge compensation (i. e. counter ion in cation uptake), activity of enzymes, photosynthesis and stomatal regulation (WELCH 1995). During cultivation of plants on solution containing $CaCl_2$, chloride ion was probably also accumulated in the vacuole (MOYA & al. 1999, SERRANO & GAXIOLA 1994), thus protecting cytoplasm of its too high concentration. Increased chloride content in vacuole can also improve osmotic adjustment of the cell. For the two lower concentrations applied, the accumulation of chloride ion in plants was directly related to chloride concentration in medium, while 0.075 mol L^{-1} did not cause further increase and after treatment with the highest concentration (0.1 mol L^{-1}) even decline of chloride content was noticed (Fig. 4). Bromide content was similar in plants treated with lower concentrations of $CaBr_2$ and mixture while concentration 0.075 mol L^{-1} of mixture, and 0.1 mol L^{-1} of both, $CaBr_2$ and mixture, caused lower accumulation of bromide (Fig. 5). Since the highest concentration of investigated solutions (0.1 mol L^{-1}) caused significantly lower accumulation of both ions, chloride and bromide (Figs. 4, 5) in comparison with treatments with lower concentrations, probably too high osmotic value disturbed normal functioning of transport across the plasma membrane.

Furthermore, in the presence of bromide, considerably lower chloride content in comparison to control plants and those treated with $CaCl_2$ has been found (Fig. 4). Since bromide belongs to the same group as chloride in the periodic system of elements, it can replace the latter in nutrient solution. Duckweed for normal growth needs 50–100 µg L^{-1} chloride that in experimental conditions could be replaced with 150 µg L^{-1} bromide (HILLMAN 1961). But in such conditions, certain degree of disturbance of physiological processes that require chloride could be expected. On the other hand, in experiments with barley roots it was established that in low-salt medium chloride absorption is competitively inhibited by bromide (WELCH 1995).

There is no much data about bromide effects on plants. Germination of 11 different crop species has not been inhibited by KBr in concentrations below 500 mg L^{-1} (4.2 mmol L^{-1}), while growth was not inhibited even at 1000 mg L^{-1} (8.4 mmol L^{-1}). In comparison to these results obtained on crop plants, duckweed showed lower sensitivity to bromide.

$CaCl_2$ and $CaBr_2$ in applied concentrations did not markedly change chlorophyll *a* and chlorophyll *b* content. Other reports showed both the inhibitory and stimulative effects of salinity on the production of photosynthetic pigments, depending on concentration, ions composition and plant species. Treatments applied in our experiment did not affect carotenoid content while in alfalfa the addition of $CaCl_2$ to medium containing NaCl increased carotenoids' content (KHAVARI-NEJAD & CHAPARZADEH 1998).

It could be concluded that investigated solutions in lower concentrations (0.025 and 0.050 mol L^{-1}) did not disturb the water content of duckweed plants while decreased fresh weight to frond number ratio and increased dry weight portion after treatment with 0.075 and 0.1 mol L^{-1} suggests increased water loss due to osmotic effect. Accumulation of chloride and bromide in plants was confirmed while calcium content was not changed. Lower accumulation of chloride in plants cultivated on medium containing $CaBr_2$ suggests that bromide could replace chloride.

Acknowledgements

We are grateful to M. TOMIĆ, M. Sc. (CROSCO Co. – Integrated Drilling and Well Services, INA-Group) for sample supply and helpful discussion in the course of this work. We would like to thank K. REMELE and Dr. G. KÖGL for help with atomic absorption spectrophotometry.

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