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Full Length Research Paper

The combined effects of cadmium and salinity on some pigments and total phenolic compounds of *Myriophyllum heterophyllum* Michx. and *Potamogeton crispus* L.

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In this study, the combined effects of different salinity and cadmium concentrations (0, 0.05‰ NaCl + 4 mg L^{-1} Cd, 0.05‰ NaCl + 64 mg L^{-1} Cd, 5‰ NaCl + 4 mg L^{-1} Cd, 5‰ NaCl + 64 mg L^{-1} Cd) on some pigments (chlorophyll a, chlorophyll b, carotenoids and anthocyanins) and total phenolic compounds were investigated in two aquatic plants (*Myriophyllum heterophyllum* Michx. and *Potamogeton crispus* L.) at 24 and 96 h. Chlorophyll a, chlorophyll b and carotenoids decreased depending on Cd + NaCl concentrations and the time (24 h and 96 h) in both species (P<0.05). The anthocyanin concentrations showed differences in combined Cd and NaCl concentrations in both species at 24 and 96 h. In the two time periods (24 and 96 h), the levels of phenolic compounds at all concentrations of Cd + NaCl increased in both *M. heterophyllum* and *P. crispus* (P<0.05).

Key words: Aquatic plants, cadmium, sodium chloride (NaCl), phenolic compounds, photosynthetic pigments.

INTRODUCTION

The heavy metals cause environmental pollution and they are among the most toxic pollutants. In addition to their toxicity effects even at low concentrations, they can be accumulated throughout the food chain, which leads to serious ecological and health hazards (Al-Rub et al., 2006). Cadmium (Cd) is a wide spread non-essential heavy metal, which enters the aquatic environments from natural (weathering of rocks) as well as anthropogenic sources (industrial effluents, agricultural run-offs). It causes oxidative stress by generating free radicals and toxic oxygen species. These species cause lipid peroxidation, membrane damage and inactivation of several enzymes by reacting with lipids, proteins, pigments and nucleic acids (Singh et al., 2005), and also cause oxidative stress (Aravind and Prassad, 2003) as well as a decrease in the content of chlorophyll and carotenoids (Shin et al., 2002). Mobin and Khan (2007) reported that anthocyanins in leaves of *Brassica juncea*, increased by cadmium stress and induction of anthocyanin accumulation, might help to protect the photosynthetic apparatus by screening it from deleterious effects of stress-generated superoxide radicals without limiting photosynthesis. Among the chemical compounds in plants, secondary metabolites have great importance in plant-environment relationships because these compounds have major ecological roles in allelophatic processes, in the protection against herbivores and in response to environmental stress of plants (Pasqualini et al., 2003). Salinity could affect the growth rate and uptake metals by plants because of the toxic effects of both sodium (Na) and chlorine (Cl) ions. Cl ions also reduce the concentration of free metal ions by forming a complex with heavy metals such as Cd and zinc (Zn). Thus, the

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toxic effects of heavy metals decrease in plants (Frittoff et al., 2005).

Sivaci et al. (2008) showed that Cd was significantly removed by *Myriophyllum heterophyllum* and *Potamogeton crispus* and Cd uptake in *P. crispus* was higher than *M. heterophyllum*. These two species are different morphologically and exhibit different biosorption capacities. They are important for polluted aquatic ecosystems because of their potential role in phytoremediation. The aquatic ecosystems are more sensitive to pollutants than terrestrial ones and aquatic plants are the first link in food chain of aquatic environments (Singh et al., 2005). Determination of physiological changes in *M. heterophyllum* (two-leaf water milfoil) and *P. crispus* (curly-leaved pondweed) due to the accumulation of the combined Cd and NaCl is important to protect the polluted aquatic ecosystems and develop several strategies in such systems. Therefore, the aim of this study is to investigate the combined effects of Cd and salinity on total chlorophyll a, chlorophyll b, carotenoids, anthocyanins and phenolic compounds in *M. heterophyllum* and *P. crispus*.

MATERIALS AND METHODS

The samples of *M. heterophyllum* and *P. crispus* were collected from Sinop Karagöl-Aksaz lagune (Black Sea Region/Turkey) and washed with diluted HCl solution (3%), followed by distilled water before use (Keskinkan et al., 2003). Analytical grade Cd sulphate ($CdSO_4$) was used as the metal source and test. Experiments were conducted at 25°C in conical flasks (250 ml cadmium and NaCl solution at varying concentrations) using an orbital shaker (200 rpm) in a constant room temperature (25 ± 2°C, 12 h light/12 h dark). Macrophyte samples (2 g wet weight) were added to each flask and placed on the orbital shaker. They were exposed to the combination of Cd and NaCl at (0, 0.05‰ + 4 mg L^{-1}, 0.05‰ + 64 mg L^{-1}, 5‰ + 4 mg L^{-1}, 5‰ + 64 mg L^{-1}). As shown by Sivaci et al. (2008), the minimum accumulation of Cd occurred in the first 24 h and maximum accumulation at 96 h in these species, and we chose this two time points to evaluate the physiological changes. After contacting periods (24 and 96 h), plant materials were filtered to separate the biomass from the solution for the determination of pigments and phenolic compounds. The experiments were performed in triplicate.

Assay for total pigments

Pre-exposure and post-exposure pigment contents of *M. heterophyllum* and *P. crispus* were measured after 24 and 96 h combined treatment with Cd and salinity. One gram of fresh leaves samples was extracted by following the method of De Kok and Graham (1989). The absorbance of the supernatants was measured at 662, 645 for chlorophylls and 470 nm for carotenoids. The concentrations were calculated as described by Lichtenthaler and Wellburn (1983).

For determination of anthocyanin, 1 g of fresh leaves samples was extracted with 12 ml of 1% (w/v) HCl in methanol for 2 days at 3 to 5°C with continuous shaking (Mancinelli et al., 1975; Reay et al., 1998). The assay was carried out in triplicate. The absorbances of samples were measured at 530 and 657 nm and anthocyanin concentrations were calculated according to using the equation by Mancinelli et al. (1975).

Assay for total phenolics

Pre-exposure and post-exposure phenolic contents of *M. heterophyllum* and *P. crispus* were measured in combination of Cd and salinity treatment after 24 and 96 h. Total phenolic constituents of macrophyte species were performed by employing the literature methods involving Folin-Ciocalteu reagent and gallic acid as standard (Slinkard ve Singleton, 1977; Chandler and Dods, 1983). Briefly, 50 mg of fresh material was homogenized in 2.5 ml ethanol and flask was kept in a water bath at 25°C for 24 h with continuous shaking. After filtration, 1 ml of supernatant solution was taken in a volumetric flask, and then 1 ml ethanol and 5 ml distilled water and 1 ml Folin-Ciocalteu reagent were added and flask was shaken thoroughly. After 3 min, 3 ml of solution 2% (w/v) Na_2CO_3 was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all standard gallic acid solutions (0-1000 µg/0.1 ml) and standard curve was obtained. Total phenolic compounds of the samples were calculated using the calibration curve with the gallic acid standard.

Statistical analysis

The data from three replications of all treatments were subjected to the analysis of variance using SPSS 8.0 for Windows. Differences between means at 5% ($P<0.05$) level were considered significant.

RESULTS

Total chlorophylls, carotenoids and anthocyanins

In *M. heterophyllum* and *P. crispus*, it was observed that the amount of chlorophyll a, chlorophyll b and carotenoids decreased by the increase of Cd + NaCl concentrations with time (24 h and 96 h) in comparison to control (Figures 1 and 2). At 24 h, the lowest level of pigment was observed in 0.05‰ + 64 mg L^{-1} in *M. heterophyllum* and *P. crispus*, (chlorophyll a, 8.32 mg g^{-1}; chlorophyll b, 2.78 mg g^{-1}; carotenoids, 2.52mg g^{-1} for *M. heterophyllum*; chlorophyll a, 8.70 mg g^{-1}; chlorophyll b, 2.94 mg g^{-1}; carotenoids 5.20 mg g^{-1} for *P. crispus*) ($P<0.05$). At 96 h, the lowest pigment contents of *M. heterophyllum* were determined in 64 mg L^{-1} + 5‰ NaCl (chlorophyll a, 7.09 mg g^{-1}; chlorophyll b, 2.02 mg g^{-1}; carotenoids; 2.10 mg g^{-1}) (Figure 1) ($P<0.05$). At 96 h, the change in pigment contents of P. crispus (chlorophyll a, chlorophyll b and carotenoids) was similar to *M. heterophyllum* (Figures 1 and 2). It was found that the decrease rate of pigment contents depending on treatment combined Cd + NaCl at 96 h was more than the 24 h in both species (Figures 1 and 2).

Moreover, in *M. heterophyllum* and *P. crispus*, there was significant changes in anthocyanin content except for 0.05‰ + 64 mg L^{-1} and 5‰ + 64 mg L^{-1} of *M. heterophyllum* at 24 h (Figure 3) ($P<0.05$). At 24 h, the highest anthocyanin concentrations were observed in 4 mg L^{-1} Cd + 5‰ NaCl in *M. heterophyllum* and 64 mg L^{-1}

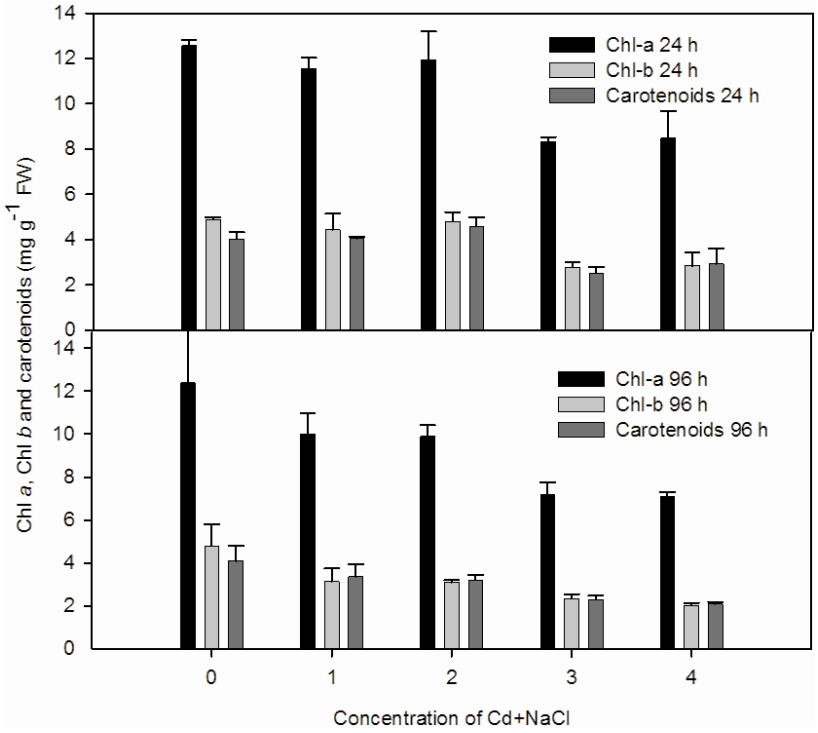

Figure 1. The combined effects of Cd and NaCl on chlorophyll a, chlorophyll b and carotenoids in *M. heterophyllum* at 24 and 96 h (0, control; 1, 4 mg L^{-1} Cd + 0.05‰ NaCl; 2, 4 mg L^{-1} Cd + 5‰ NaCl; 3, 64 mg L^{-1} Cd + 0.05‰ NaCl; 4, 64 mg L^{-1} Cd + 5‰ NaCl) (chlorophyll a, Chl a; chlorophyll b, Chl b).

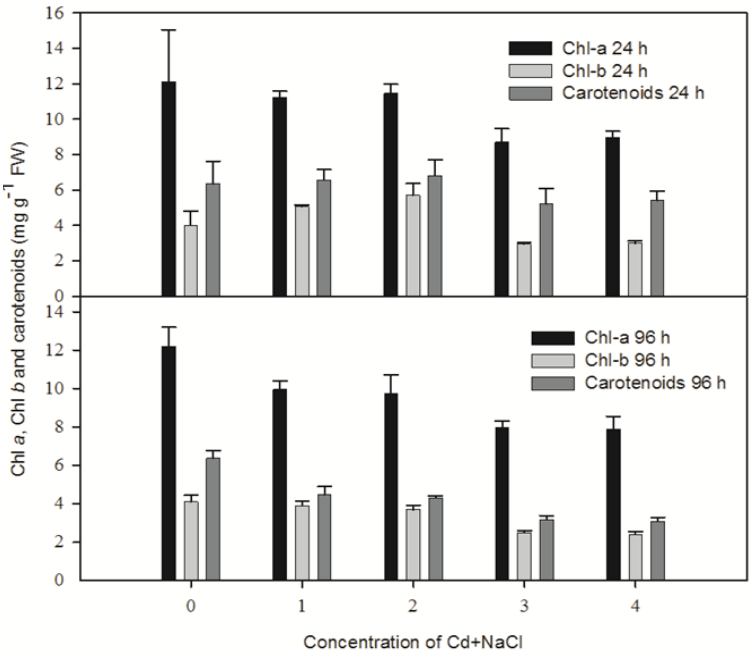

Figure 2. The combined effects of Cd and NaCl on chlorophyll a, chlorophyll b and carotenoids in *P. crispus* at 24 and 96 h (0, control; 1, 4 mg L-1 Cd + 0.05‰ NaCl; 2, 4 mg L-1 Cd + 5‰ NaCl; 3, 64 mg L-1 Cd + 0.05‰ NaCl; 4, 64 mg L-1 Cd + 5‰ NaCl) (chlorophyll a, Chl a; chlorophyll b, Chl b).

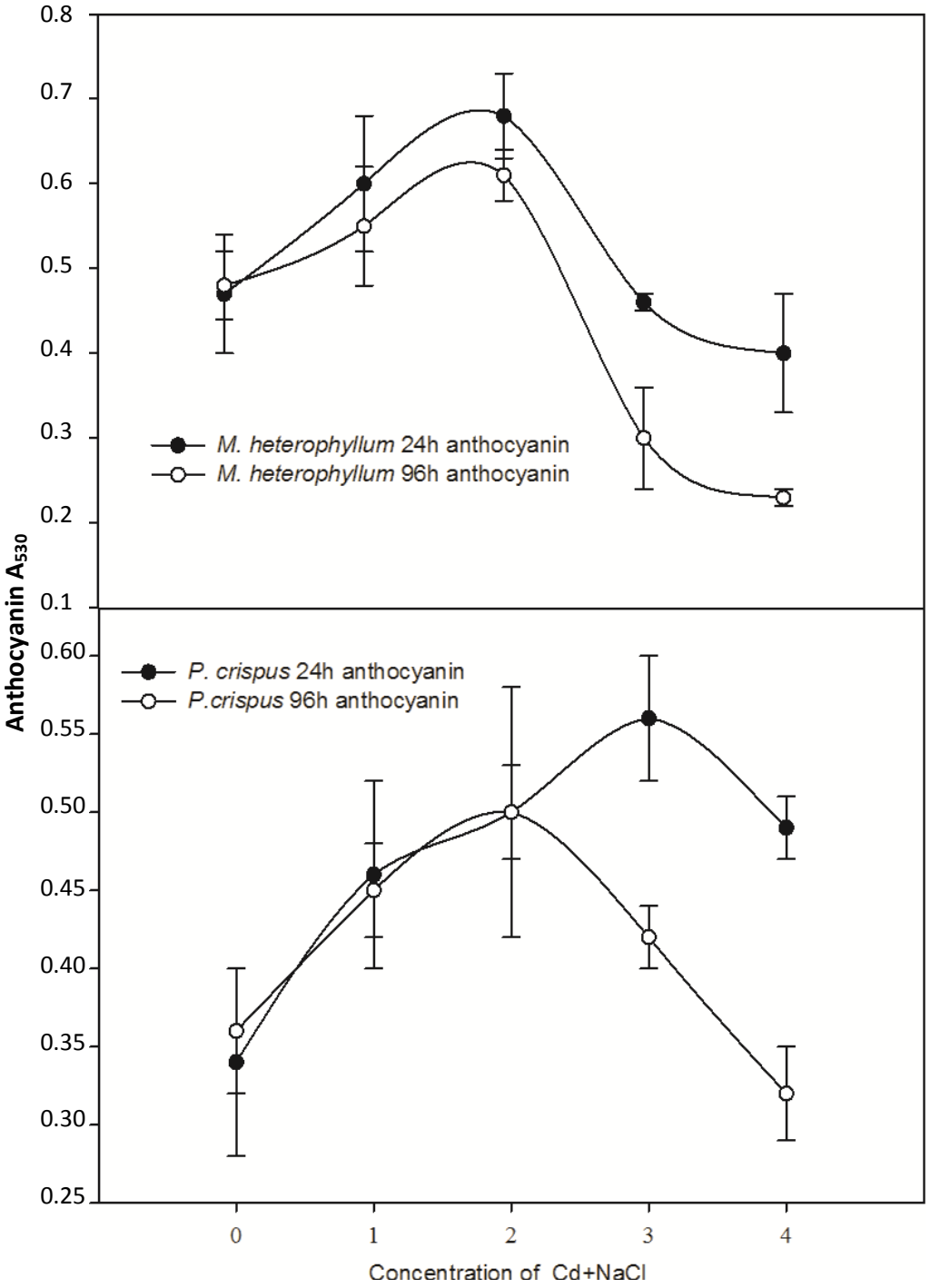

Figure 3. The combined effects of Cd and NaCl on anthocyanins in *M. heterophllum and P.crispus* at 24 and 96 h (0, control; 1, 4 mg L^{-1} Cd + 0.05‰ NaCl; 2, 4 mg L^{-1} Cd + 5‰ NaCl; 3, 64 mg L^{-1} Cd + 0.05‰ NaCl; 4, 64 mg L^{-1} Cd + 5‰ NaCl).

Cd + 0.05‰ in *P. crispus* (Figure 3). At 96 h, the highest anthocyanin concentrations were determined in 4 mg L^{-1} Cd + 5‰ NaCl ($P<0.05$) and the lowest in 64 mg L^{-1} Cd + 5‰ NaCl in *M. heterophyllum* ($P<0.05$) and *P. crispus* ($P>0.05$) (Figure 3).

Total phenolics

The total phenolic compounds increased with increasing Cd + NaCl concentrations in both treatment periods (24 h and 96 h). In both *M. heterophyllum* and *P. crispus*, the

Table. 1. The combined effects of Cd and NaCl on total phenolic compounds in *M. heterophyllum* and *P. crispus* at 24 and 96 h. Each value indicates the mean ± standard deviation (µg / mg FW).

Treatments	*Myriophyllum heteropyhllum*		*Potamogeton crispus*	
	24 h	96 h	24 h	96 h
Control	55.62 ± 2.67	57.10 ± 8.00	67.37 ± 6.70	69.83 ± 5.56
4 (mg/L) Cd + 0.05‰ NaCl	61.00 ± 0.50	63.80 ± 4.10	71.68 ± 6.80	73.09 ± 4.58
4 (mg/L) Cd + 5‰ NaCl	62.00 ± 1.20	64.40 ± 5.20	72.20 ± 4.44	73.86 ± 4.33
64 (mg/L) Cd + 0.05‰ NaCl	73.30 ± 0.34	76.18 ± 0.97	80.50 ± 4.56	83.68 ± 6.70
64 (mg/L) Cd + 5‰ NaCl	74.70 ± 0.23	77.21 ± 5.38	82.00 ± 5.21	86.70 ± 12.00

highest level of phenolic compound was found in 64 mg L^{-1} Cd + 5‰ NaCl at 24 h and 96 h (Table 1) ($P<0.05$). Meanwhile, the level of phenolic compounds was higher in *P. crispus* than *M. heterophyllum* (Table 1).

DISCUSSION

In the present study, the contents of chlorophyll a, chlorophyll b and carotenoids decreased due to the time and the increasing concentrations of Cd + NaCl in both species (Figures 1 and 2). Photosynthetic activity of plants can decrease under various stress conditions (Santos, 2004; Singh et al., 2005; Mobin and Khan, 2007; Sivaci et al., 2004, 2008). Garg and Singla (2004) reported that the chlorophyll concentrations were decreased by salt stress in chickpea cultivars. Rai et al. (2005) reported that increasing Cd concentration caused a decrease in the concentrations of chlorophyll a, chlorophyll b and carotenoids in *Phyllanthus amarus* and chlorophyll b was more sensitive than chlorophyll a. In another study, pigment contents in leaves of *Alternanthera philoxeroides* (chlorophyll a, chlorophyll b and carotenoids) decreased by increasing the Cd gradient concentrations (Ding et al., 2007). Frittoff et al. (2005) determined that the toxic effect of Cd decreased due to forming complexes of the chloride ions with heavy metals such as Zn and Cd under high salinity in *Elodea canadensis* and *P. natans*. In *Zea mays*, the chlorophyll content decreased at various levels of salinity or following Cd treatment. In contrast, there was an increase in chlorophylls in combined Cd and NaCl according to the report of Sepehr and Ghorbanli (2006). When compared the results of the present study and Sıvacı et al. (2008), it was found that the decrease of chlorophyll a, chlorophyll b and carotenoids contents in combined effect with Cd +NaCl was lesser than the samples treated with Cd alone in the two macrophytes at 24 h. Hale et al. (2001) reported that molybdenum was accumulated in peripheral cell layer probably forming a complex with anthocyanin in *Brassica rapa* and the levels of anthocyanin were in concordant with Mo concentration. In another study, an increase in anthocyanin was observed in *B. juncea* cultivars with Cd stress and these findings indicated that the accumulation of anthocyanin may be only of secondary importance in living cells (Mobin and Khan, 2007). As mentioned in these studies, we determined an increase in the anthocyanin contents of two macrophytes at the some combination of Cd and NaCl concentrations by passage of time (Figure 3).

The plants can accumulate phenolic compound in their organs due to biotic and abiotic stress (Draper, 1997). Ksouri et al. (2007) investigated the effects of salinity on phenolic compounds in *Cakile maritima* which grows in different regions. In their study, there was a variation in the accumulation of phenolic compounds in the species due to salinity, as to genetic structure and environment. According to Ferrat et al. (2003), biotic/abiotic stress induces the synthesis of polipherol and accumulation of these compounds in plants. Also, phenolic compounds have intra- and inter-specific competition capacity and the most make a significant contribution to antioxidant activity of plants by bounding the heavy metals. In another study about *Myriophyllum spicatum* and *Myriophyllum triphyllum,* Sivaci et al. (2007) found that Cd affected the phenolic compounds at certain concentrations in both species and this effect changed as to species. In our study, the content of phenolic compounds increased depending on combination of Cd and NaCl concentrations in the two species and this corresponds with the investigations aforementioned. This result showed that plants may be accumulating the phenolic compounds under various stress conditions.

Conclusion

The level of pigments (chlorophyll a, chlorophyll b, carotenoids and anthocyanin) and phenolic compounds changed depending on the time (24 and 96 h) in stressed aquatic plants. According to the obtained results, chlorophyll a, chlorophyll b and carotenoids contents of the two aquatic plants decreased at all Cd + NaCl concentrations by time (24 and 96 h), but anthocyanin concentrations increased in some concentrations. Also, total phenolic compounds in these two aquatic plants increased with increasing Cd + NaCl concentrations in both treatment periods (24 and 96 h).

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