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RESEARCH ARTICLE

Influence of exogenous urea on photosynthetic pigments, $^{14}CO_2$ uptake, and urease activity in *Elodea densa*—environmental implications

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Abstract This paper analyzes the effect of exogenous urea in increased concentration gradient (0, 100, 500 and 1,000 mg L^{-1}) on photosynthetic pigments (measured spectrophotometrically), uptake of $^{14}CO_2$ (using radioisotope), and urease activity (by measuring ammonia with Nessler's reagent) in leaves of *Elodea densa* Planch. We have observed that low concentration of urea (100 mg L^{-1}) stimulates the accumulation of photosynthetic pigments and intensifies photosynthesis in *E. densa*, whereas high concentration (1,000 mg L^{-1}) suppresses these processes. Urease activity increased by approximately 2.7 and 8 fold when exogenous urea concentrations were 100 and 500 mg L^{-1}, respectively. However, exogenous urea in high concentration (1,000 mg L^{-1}) decreased urease activity by 1.5 fold compared to the control. The necessity of mitigating urea and other nitrogen-containing compounds (NH_3 from urea) in water bodies has been discussed with emphasis on the potential for phytoremediation of urea using common water weed viz. *E. densa*.

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Introduction

Anthropogenic and climate change phenomena increased pollution loads in water bodies. Contemporary agrarian activity is also responsible for causing environmental imbalance by pesticide residues and toxic agrochemical accumulation in water bodies, soils, and sediments. Therefore, watershed runoff management and mitigation measures are priority for research. Most of the excess nutrients from agroecosystems (nitrogenous and phosphorus), including a variety of pollutants from non-point sources, get washed away into water bodies. Other anthropogenic sources such as sewage and domestic waste discharged into aquatic systems accelerate eutrophication. In recent years, the problem of aquatic pollutants coming from catchment areas is increasing significantly. Urea (carbamide), a commonly used fertilizer, is one of the predominant pollutants (Bremner and Krogmeier 1988). Urea is often used as a nitrogen fertilizer not only to improve the soil fertility but also to increase the productivity of fish ponds (Herbeck et al. 2012). Therefore, substantial amounts of urea enter water bodies because of anthropogenic activity.

Much of the urea gets discharged into rivers and reservoirs during periods of heavy snowmelt and high rainfall. For high water hydrological phases (spring floods and summer rainstorm runoff), agricultural lands contribute 70 % of the total water pollution from runoff (Borisova and Feudorova 1999). Inflow of sewage during the spring and summer flash floods from livestock farms also poses serious environmental implications.

Nitrogen from urea enters the plant either directly or in the form of ammonium or nitrate after microbial degradation. Urea metabolism in plants has been recently reviewed by Witte (2011). Urea is also produced by plants, fungi, bacteria, and other aquatic organisms as a product of ammonification of proteins. In addition, it is also formed during the decay of dead organisms as a result of microbial decomposition of purine and pyrimidine bases. The decomposition of urea in the aquatic environment is carried out mainly by the enzymes of bacteria and plants, especially urease—a nickel-dependent metalloenzyme (EC 3.5.1.5, urea amidohydrolase) (Strock 2008). Urease is present in many living organisms, including higher and lower plants and some species of bacteria and fungi. Urease catalyzes the reaction of the hydrolytic decomposition of urea into ammonia and carbon dioxide during the vital activity of organisms and for some time after their death (Baker and Thompson 1962; Sirko and Brodzik 2000). Ammonia which is formed during the hydrolysis of urea may participate in the amination of keto acids or may oxidize to nitrites and nitrates.

Urea is selected as an additive for rapid removal of 2,4,6-trinitrotoluene (TNT) from soil by vetiver grass (Das et al. 2010). In such instances, urea serves as an additional source of carbon and nitrogen. This is also true in case of submerged aquatic plants where light and concentrations of inorganic carbon are limiting factors. At the same time, influx of urea into water bodies is reported to change the water quality and the species composition of the population (Azizullah et al. 2011; Gonzalez and Plamondon 1976–1977; Ng et al. 1990). It is known that high doses of urea and its concomitant hydrolyzed products such as ammonia are implicated in physiological disturbances and loss of productivity of higher plants, bringing changes in dominant species in plant communities (Usenko et al. 2000). It is also reported that foliar applications of urea caused phytotoxicity (Krogmeier et al. 1989). However, information about the influence of exogenous urea on the photosynthetic functions of *Elodea densa*, the most common aquatic weed in water bodies all over the world, is rather scanty. Further, plant metabolism has been applied for phytoremediation of agrochemicals (Coleman et al. 2002; Chaudhry et al. 2002; Dosnon-Olette et al. 2011; Gatidou and Iatrou 2011).

Therefore, the objectives of the present study are (a) to investigate the changes in the structure and functioning of the photosynthetic apparatus of *E. densa* in the presence of exogenous urea in different doses (low and high), (b) to study the uptake of $^{14}CO_2$ as a function of photosynthesis in different concentrations of exogenous urea, and (c) to assay the urease activity and protein content in *E. densa* leaves treated with urea in different concentrations.

Materials and methods

Plant material and urea treatment

Experiments were performed with *E. densa* Planch (Hydrocharitaceae), a submerged hydrophyte (waterweed). Its leaf blades are thin consisting of only two cell layers—the upper and lower epidermis, which perform the main photosynthetic function. This species can be cultivated in laboratory conditions as the plants grow without rooting, producing large biomass. Because of these properties, elodea plants are often used as a model hydrophyte for toxicity bioassays. *Elodea* shoots measuring 6–10 cm in length were incubated for 5 days on 5 % Hoagland–Arnon I nutrient solution (1 L of medium contained 41 mg of dehydrated $Ca(NO_3)_2$, 25 mg of KNO_3, 6.8 mg of KH_2PO_4, and 12 mg of $MgSO_4 \times 7\ H_2O$) with urea in the increased concentration gradient: 0 (control), 100, 500, and 1,000 mg L^{-1}. Samples were incubated at 23–25 °C under natural illumination.

Measurement of photosynthetic pigments

For photosynthetic pigment analysis, each leaf sample (30 mg) was homogenized in cooled 80 % acetone and centrifuged for 10 min at 5,000 ×*g*. The absorbance of the supernatants was measured at wavelengths 470, 646, and 664 nm with a PD-303 UV spectrophotometer (Apel, Japan). The content of chlorophylls (*a* and *b*) and carotenoids was calculated according to Lichtenthaler (1987).

Measurement of CO_2 uptake

The intensity of potential photosynthesis in *Elodea* was determined by radioisotope method (Mokronosov and Dobrov 1973) under illuminance of 40 klx at 23 °C and 0.4 % $^{14}CO_2$ in the air. The leaves were amply moistened during measurements. The duration of exposure to $^{14}CO_2$ for all determinations was 5 min. The specific radioactivity in the experiments was 1 GBq L^{-1} $^{14}CO_2$. Radiometric measurements were accomplished with a 20046 Veb Robotron-Messelektronik radiometer (Germany).

Urease assay

Urease (EC 3.5.1.5) was assayed by measuring ammonia using Nessler's reagent according to Jayaraman (1981). *Elodea* leaves (0.5 g) were homogenized in 5 mL of cold sodium–phosphate buffer (0.1 M, pH 7.4), containing 1 mM Na EDTA and 2 % PVP. The homogenate was centrifuged in 8,000 rpm for 10 min. The supernatant was collected, and the urease activity was measured after the reaction with Nessler's reagent at 405 nm. The amount of ammonia liberated in the test solution was calculated by calibrating the

reagent with standard ammonium chloride solution. The urease activity was expressed as μmoles NH_3 mg^{-1} of protein per minute.

Protein assay

The protein concentration was determined according to Bradford (1976), using bovine serum albumin (Sigma) as a standard.

Statistics

All experiments were performed in three replications, each of which is represented by a sample of 10–15 plants. Significance of difference between the variants was determined by the non-parametric Mann–Whitney *U* test with a significance level of $p<0.05$. The figures show the arithmetic means of the three biological replicates and their standard errors. Different letters present significantly different values.

Results

Urea in lower dose (100 mg L^{-1}) in the culture medium stimulated the formation of chlorophyll *a*, carotenoids, and $^{14}CO_2$ uptake, showing increased values by 15–30 %, respectively (Figs. 1a–c and 3). With increasing concentrations of urea up to 500 mg L^{-1}, content of photosynthetic pigments decreased but remained above the control (Fig. 1a, b). At maximum dose of urea (1,000 mg L^{-1}), the amount of total chlorophyll (*a*+*b*) decreased significantly lower than that of the controls (Fig. 1a, b). In this case, the content of carotenoids also decreased, but not significantly (Fig. 1c), dependent on the urea dose.

The chlorophyll *a*/*b* ratio steadily increased dependently on urea dose (Fig. 2a). The ratio of the amount of chlorophylls (*a*+*b*) to the carotenoids changed insignificantly (Fig. 2b), but tended to decrease.

At higher concentrations of urea, the rate of $^{14}CO_2$ uptake decreased sharply (Fig. 3). In the presence of urea at a concentration of 1,000 mg L^{-1} the rate of photosynthesis was three times lower compared with that of the control.

In our experiments, we observed a direct correlation between the exogenous urea and urease activity up to 500 mg L^{-1}. The urease activity increased 2.7 fold when the urea concentration was 100 mg L^{-1} and increased approximately 8 fold when the exogenous urea in the medium was 500 mg L^{-1} (Table 1). However, the high concentration (1,000 mg L^{-1}) of exogenous urea reduced the urease activity 1.5 times lower than that of the control. Interestingly, the protein concentration remained stable, independent of the concentrations of exogenous urea.

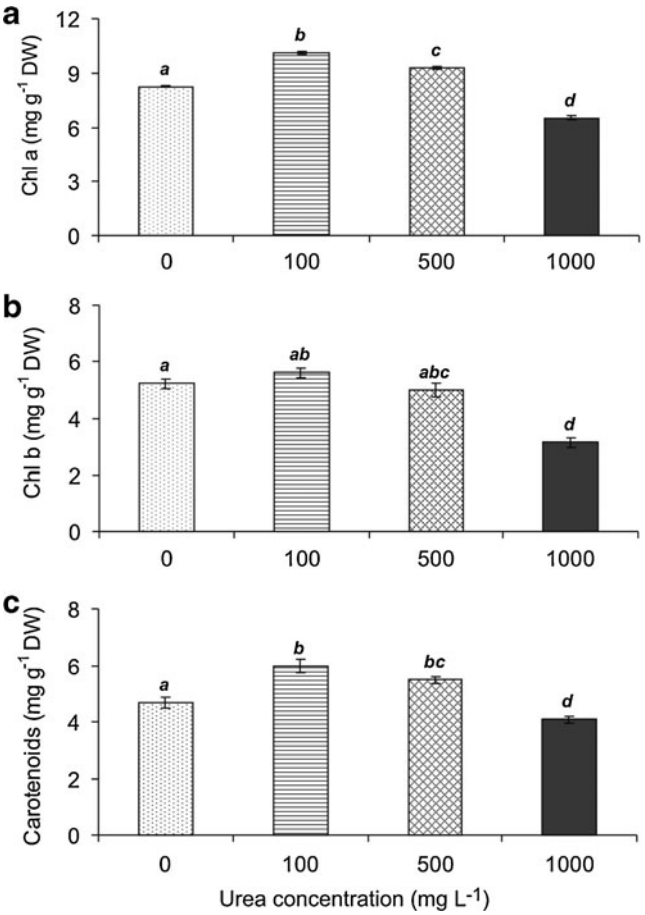

Fig. 1 Effect of different urea concentrations on chlorophyll *a* (**a**), chlorophyll *b* (**b**), and carotenoid (**c**) content in *E. densa* leaves. Data represent means ± SE ($n=3$). Means within each graph followed by the same letters do not differ statistically according to Mann–Whitney *U* test ($p\leq0.05$)

Discussion

Removal of pollutants from waste waters by efficient and low cost methods, which use renewable energy sources, is relevant in the contemporary era of climate change. One such approach is phytoremediation using rapid high-biomass producing plants, which use natural solar energy to stabilize, remove, degrade, or otherwise deal with contaminants (Prasad 2004; Rai 2009). *E. densa* is one ideal example, as it grows all over the world and produces a large biomass (Mony et al. 2007; Maleva et al. 2012).

Photosynthesis is one of the leading functions of plants. Changes in the structural and functional parameters of the photosynthetic apparatus can be an adaptation to unfavorable conditions (Malec et al. 2010; Saygideger et al. 2009). In our experiments with different doses of urea (100, 500, and 1,000 mg L^{-1}), we have observed that the trend of photosynthesis coincided with the changing dynamics of photosynthetic pigments (Figs. 1a–c and 3). High doses of urea led to the suppression of photosynthetic function in

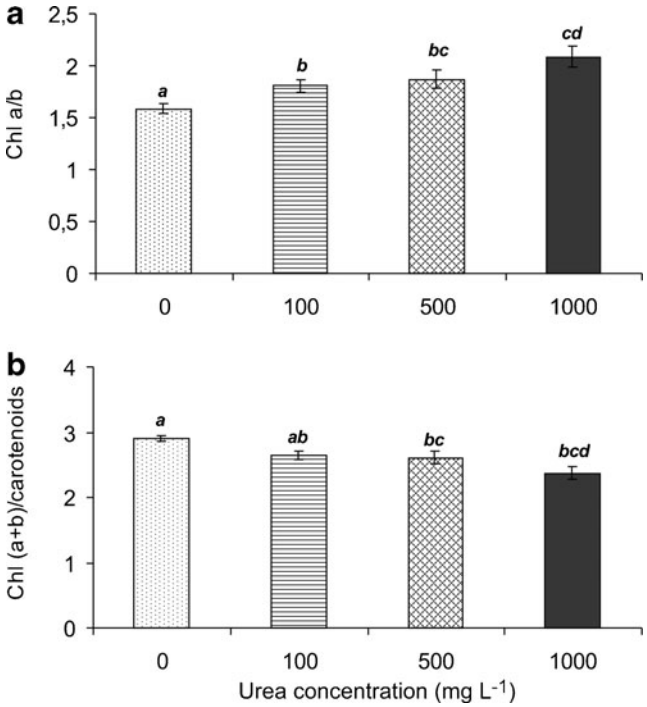

Fig. 2 Effect of different urea concentrations on chlorophyll *a*/*b* ratio (**a**) and chlorophyll (*a*+*b*)/carotenoids ratio (**b**) in *E. densa* leaves. Data represent means ± SE (n=3). Means within each graph followed by the same letters do not differ statistically according to Mann–Whitney *U* test ($p \leq 0.05$)

plants. It is known that high concentrations of urea cause the oxidation of organic molecules and damage the cell membranes (D'Apolito et al. 2010). Obviously, the destruction of chlorophylls and carotenoids by oxidative stress was the main reason for the decrease of functional activity of the photosynthetic apparatus. This is confirmed by the calculated Spearman rank correlation coefficients between the content of photosynthetic pigments and the potential intensity of photosynthesis (Table 2).

The ratios of chlorophyll *a*/*b* and total chlorophyll (*a*+*b*) /carotenoids are significant indicators for the resistance of

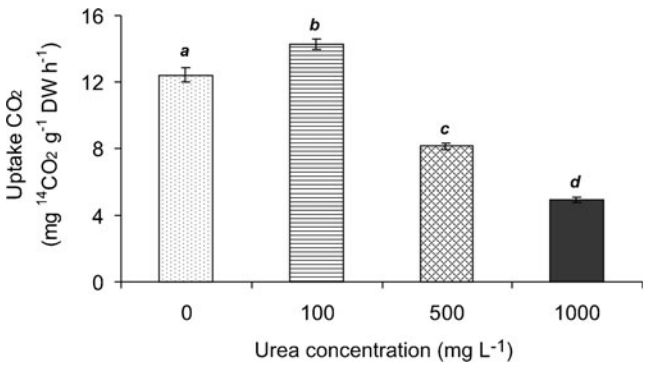

Fig. 3 Photosynthetic rates in *E. densa* leaves treated with different concentrations of urea for 5 days. Data represent means ± SE (n=3). Means within each graph followed by the same letters do not differ statistically according to Mann–Whitney *U* test ($p \leq 0.05$)

pigment complexes to different pollutants. These ratios are normally quite stable but change rapidly with various stress factors (Maleva et al. 2012). Changes in the proportion of photosynthetic pigments are reflected in the activity of the photosynthetic apparatus, thus affecting the rate of assimilation as well as plant growth and ultimately productivity. The fact that chlorophyll *a*/*b* ratio significantly increased independently on urea dose suggests that chlorophyll *a* is more stable than chlorophyll *b*. The decrease in the ratio of chlorophyll (*a*+*b*) to carotenoids indicates the stability of the latter to stress by high concentrations of urea.

The observed trends in the responses of *E. densa* to the effect of urea differ from the data obtained earlier (Maleva et al. 2012), reflecting the change in the ratio of photosynthetic pigments in plants of this type under the action of heavy metals. According to these studies, chlorophyll *a* was more sensitive to the combined effect of Cu and Mn in comparison with chlorophyll *b*, and the stability of chlorophylls was higher than that of carotenoids.

The increase in carotenoids in the presence of urea (100 and 500 mg L^{-1}) is explained by the ability of carotenoids to interact with reactive oxygen species, inhibiting the process of their accumulation, and thus preventing oxidative stress. It is known that carotenoids are lipophilic antioxidants, the most effective antioxidants for extinguishing the excess energy of triplet chlorophyll and singlet oxygen (Chirkova 1997).

Urea assimilation and metabolism in plants have gained considerable attention (Witte 2011). Hydrolysis is the main metabolic process for the breakdown of urea, and subsequently, urea is assimilated through carbon and nitrogen cycles. A relationship between chlorophyll and nitrogen content of North American forest tree species was determined by van den Berg and Perkins (2004). Using a portable chlorophyll meter, chlorophyll (Chl) and nitrogen (N) contents in sugar maple (*Acer saccharum* Marsh) leaves were estimated. A significant correlation between chlorophyll content index values and extractable Chl and N in large heterogeneous samples was observed (van den Berg and Perkins 2004). In addition, using a portable chlorophyll meter, nutritional status of tree crops has been predicted, particularly nitrogen, magnesium, and iron (Shaahan et al. 1999). Nitrogen nutrition was also considered to be an indicator of winter wheat based chlorophyll content and leaf area index (Houlès et al. 2007).

Increased amounts of chlorophylls in *Elodea* leaves with increasing concentration of exogenous urea indicate a relationship between Chl and N (Houlès et al. 2007; Shaahan et al. 1999; van den Berg and Perkins 2004). This observation is strengthened by the studies on Chl and N. Therefore, the addition of urea in small quantities has led to increased synthesis of chlorophyll.

Studies have shown that the contents of photosynthetic pigments in *Elodea* leaves were significantly dependent on

Table 1 Urease activity and protein content in *E. densa* leaves treated with different concentrations of urea for 5 days

Urea concentration (mg L^{-1})	Urease activity		Protein content	
	μM NH_3 mg^{-1} protein min^{-1}	% of control	mg g^{-1} DW	% of control
0 (control)	0.029±0.002 a	100	166±2.33 a	100
100	0.079±0.005 b	270	173±2.43 a	104
500	0.233±0.006 c	796	188±1.72 b	114
1,000	0.019±0.001 d	65	172±1.89 a	104

Data represent means ± SE (n=3). Means within each row followed by the same letters do not differ statistically according to Mann–Whitney U test ($p \leq 0.05$)

DW dry weight

the concentration of urea in solution. This suggests that the *Elodea* leaves easily absorbed exogenous urea. It is known that urea can be accumulated by the plant roots themselves and assimilate into root cells, but high concentrations of urea cause denaturation of protein molecules (Lima et al. 2009; Rossky 2008). Urea is able to interact with both polar and non-polar groups of proteins, leading to the destruction of their tertiary structure. It was found that urea can directly break the hydrogen bonds in protein molecules. Therefore, exogenous urea can be considered as a stress factor for plants, since substantial amounts of urea and other nitrogen-containing substances come into water bodies from rural watersheds. Nutrient discharge into water bodies depends on the intensity of anthropogenic activity in rural watersheds. Maximum concentrations of nitrogen (both ammonium and nitrate) were observed in the runoff from the plowed lands (Borisova and Konistyapina 2002).

Urea is rapidly hydrolyzed with the assistance of soil bacteria (Witte 2011). To prevent the accumulation of urea and its nitrogenous components in surface waters, mitigating measures are needed, aiming at reducing the quantity of nitrogen-containing compounds in runoff. Compliant with the requirements for environmentally sound use of urea as a fertilizer, disposal and recycling of waste by phytoremediation measures to prevent the entry wastewaters can significantly reduce the flow of urea and other nitrogen-containing substances into aquatic ecosystems.

Table 2 Coefficients of Spearman rank correlation between the intensity of potential photosynthesis and the content of photosynthetic pigments in leaves of *E. densa*, incubated for 5 days in medium with different concentrations of urea

Spearman correlations (n=12)	Chl *a*	Chl *b*	Chl (a+b)	Carotenoids
Uptake CO_2	0.76 *p=0.0039*	0.83 *p=0.0006*	0.78 *p=0.0025*	0.62 *p=0.031*

Chl chlorophyll

Maximum permissible concentration of urea for fishing water bodies in Russia is 80 mg L^{-1} (Bespamyatnov and Krotov 1985). High doses of urea and its concomitant hydrolyzed products such as ammonia are toxic, causing water pollution in addition to bringing changes in dominant species in plant communities (Usenko et al. 2000). The urea solution at a concentration of about 1.5 % was reported to be toxic to potato leaves, and at a concentration between 5 and 10 % was found to be lethal (Mokronosov et al. 1966).

In our experiments, the urease activity increased sharply (eight times), but only at concentrations of urea up to 500 mg L^{-1} (Table 2). In the high concentration treatment (1,000 mg L^{-1}), the urease activity was reduced to 1.5 times compared to that of the control. It is presumed that in high doses of exogenous urea, *Elodea* leaves tend to accumulate toxic ammonia that causes inactivating urease function by "feedback inhibition".

Conclusions

Our studies which aim to evaluate the influence of exogenous urea on the photosynthetic activity of *E. densa* showed that the presence of urea in the incubation medium at low concentrations (up to 500 mg L^{-1}) stimulated the synthesis of photosynthetic pigments, $^{14}CO_2$ uptake, and urease activity. On the other hand, 1,000 mg L^{-1} was toxic. Thus, excessive use of urea can negatively influence aquatic ecosystems, necessitating the development of phytotechnological interventions to curtail this influx. In this direction, the study of macrophytes structural and functional parameters and their effective use for biological monitoring and phytoremediation of water bodies to improve the environmental state of aquatic ecosystems is highly desirable.

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