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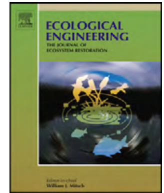

Physiological responses of *Phragmites australis* to wastewater with different chemical oxygen demands

Jingtao Xu[a], Jian Zhang[a,*], Huijun Xie[b], Cong Li[a], Nan Bao[a], Chenglu Zhang[a], Qianqian Shi[a]

[a] Shandong Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Shandong University, Jinan 250100, China
[b] Environmental Research Institute, Shandong University, Jinan 250100, China

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ABSTRACT

Constructed wetlands have been widely used to treat various wastewaters with large differences in their concentration of pollutants. The capability of wetland plants to resist these wastewaters is crucial for a wetland's healthy development. *Phragmites australis* has been shown to have the capability to grow in simulated wastewater containing a wide concentration of pollutants. In this study, the physiological responses of *P. australis* to simulated wastewaters with high chemical oxygen demands (CODs) were investigated in a bucket experiment. *P. australis* was incubated in buckets for 30 days at five treatments of 0, 100, 200, 400, and 800 mg L^{-1} COD simulated wastewater. The net photosynthesis rate of the plants declined markedly with increasing COD levels. Proline and malondialdehyde (MDA) contents also increased dramatically. The plants further showed a unimodal pattern of superoxide dismutase (SOD) and peroxidase (POD) distribution along external COD values on the whole, indicating that high COD values ($\geq$200 mg L^{-1}) can disrupt the normal metabolism of the plant. High COD levels (COD $\geq$ 400 mg L^{-1}) caused evident physiological changes in *P. australis*.

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1. Introduction

Constructed wetlands are an effective method to remove organic matter. They are easily operated and maintained, require low input, and consume less energy, which is especially appropriate for developing countries (Buchberger and Shaw, 1995; Haberl, 1999; Kivaisi, 2001; Solano et al., 2004). Constructed wetlands have been widely used in various wastewater treatment processes, including domestic and industrial wastewater, acid mine drainage, agricultural runoff, and landfill leachate treatment (Kadlec and Knight, 1996; Scholz, 2006). However, the extreme conditions found in wastewater treatment systems influence plants to a large extent (Karatas et al., 2009) and even exceed the tolerance of aquatic plants (Surrency, 1993), limiting both plant survival and treatment potential. The capacity of wetland plants to resist these wastewaters is crucial for a wetland's healthy development. Wetland plants play an essential role in constructed wetlands (Brix, 1987; Reed et al., 1988; Clarke and Baldwin, 2002), improving the pollutant removal capacity of wetland systems. Thus, the selection of wetland plants is an important step in wetland construction. One of the criteria in plant selection is the plant's strong resistance to varied concentrations of pollutants in different samples of wastewater.

Plant stress from pollutants has been studied for many years and has been extensively reviewed. Many studies have been performed regarding the effects of heavy metals, such as aluminum (Rout et al., 2001), cadmium (Benavides et al., 2005), and zinc (Broadley et al., 2007), in plants, and their resistance mechanisms have been reviewed. Plants have been found to be capable of accumulating a certain amount of heavy metal. Excessive levels of heavy metals, however, will inhibit their growth, damage their structure, affect their physiological and biochemical activities, and decrease their functions. The responses of plants to increased concentrations of ammonia have been reviewed by Britto and Kronzucker (2002), who discussed the different effects of ammonia on the growth, survival, physiology, and biochemistry of plants. The effects of salinity on plants and resistance mechanisms have been reviewed by Munns and Tester (2008), who discussed responses to salinity stress and the mechanisms of salinity tolerance at the molecular, cellular, and whole plant levels. Armstrong et al. (1996) studied the effect of phytotoxins on *Phragmites australis*, suggesting that accumulated phytotoxins would retard the recovery of the plant from stress. However, no work has so far been carried out to study the responses and tolerance of plants to increased CODs in wastewater.

Chemical oxygen demand (COD) is a measure of the capacity of water to consume oxygen (O_2) during the decomposition of organic matter and the oxidation of inorganic chemicals, such as ammonia

* Corresponding author. Tel.: +86 531 8836 3015; fax: +86 531 8836 4513.
E-mail address: zhangjian00@sdu.edu.cn (J. Zhang).

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and nitrites. The higher the value of COD, the lower the dissolved oxygen in water. When the water oxygen content is lower than normal, plants can experience either hypoxic stress, a condition of low O_2, or anoxic stress, a complete deprivation of O_2. Oxygen deprivation has many negative impacts on the growth and metabolism of plants. It creates hypoxia or anoxia around plant tissues like root apices and the stele (Collis and Melville, 1974; Kennedy et al., 1992), even if aerenchyma is present in the stele (Armstrong and Beckett, 1987; Thomson and Greenway, 1991). In this condition, respiration shifts to anaerobic mode, and alcoholic fermentation occurs in the plants (Das and Uchimiya, 2002).

Oxygen stress can be induced by oxygen deprivation. Under oxidative stress, the generation of reactive oxygen species can cause damage to plants, such as the degradation of proteins, peroxidation of lipids, and induction of antioxidant enzymes activity (Blukhin et al., 2003; Pourabdal et al., 2008). In plants, a deficiency in oxygen dramatically reduces the efficiency of cellular ATP production, which has diverse ramifications for cellular metabolism and developmental processes (Fukao and Bailey-Serres, 2004). Reactive species induce the peroxidation of lipids in the plasmalemma and intracellular organelles, which leads to an outflow of cellular contents, affects respiratory activity in mitochondria, and causes a loss of the carbon-fixing capability of chloroplasts (Scandalios, 1993). To resist created oxidative stress, plants have a highly efficient protective system that can obliterate, neutralize, or sweep these reactive oxygen species. The enzymatic defense system, one of the defense systems of plants, includes antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbic peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Asada and Takahashi, 1987; Yordanova et al., 2003). High COD wastewater may induce osmotic stress in plants, similar to the way high concentrations of salt cause hyperosmotic stress, and immediately affect growth, reduce cell expansion in root tips and young leaves, and cause stomatal closure. Proline is thought to play a cardinal role as an osmoregulatory solute in plants subjected to hyperosmotic stresses (Delauney and Verma, 1993). Proline, which is distributed in the cytoplasm as a protective material, is the most important and effective osmotic adjustable organic substance in plants. It maintains the balance between the protoplast and the external environment and prevents cells' loss of water.

Wetlands are an effective method for removing COD in wastewater. However, wastewater COD values vary greatly. Even in the same type of wastewater, COD values may sometimes differ by several times or even several dozen times. Thus, it is very important to study the response regularity and tolerance of plants to CODs. The present study aims to analyze the response and tolerance to increased CODs of a wetland plant, *P. australis*, which is a well-developed underground creeping rhizome, and the most commonly used plant in constructed wetlands (Vymazal and Krőpfelová, 2005). However, at the sewage purification stage, wastewater could cause counterproductive effects in plants and even kill them. The health of the wetland could also be affected. In this paper, the changes in the physical characteristics of reeds were analyzed after the reeds were subjected to stress provided by different concentrations of wastewater over 30 days. Determination of the net photosynthetic rate, antioxidant enzymes, proline, MDA and dissolved oxygen, and pH was carried out.

2. Materials and methods

2.1. Plant culture and treatments

P. australis was transplanted uniformly from Nansihu Lake in May of 2008 and precultured outdoors in plastic buckets 45 cm in depth and 37 cm in diameter under a transparent roof. Each of the treatment buckets contained five plants, 25 cm sand (particle size ≤2 mm, collected from Yellow River), and 15 cm tap water. To allow drainage of water, a 2-cm diameter hole was drilled at the bottom of each bucket. Prior to the experiments, the plants were cultured for 2 weeks with tap water and then were cultured for 2 weeks in 10% Hoagland solution (Hoagland and Arnon, 1950) for acclimatization. The 10% Hoagland solution had the following composition: 0.6 mM KNO_3, 0.4 mM $(CaNO_3)_2{\cdot}4H_2O$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4{\cdot}7H_2O$, 0.9 μM $MnCl_2{\cdot}4H_2O$, 0.46 μM H_3BO_2, 0.08 μM $ZnSO_4{\cdot}7H_2O$, 0.03 μM $CuSO_4{\cdot}5H_2O$, 0.01 μM $H_2MoO_2{\cdot}H_2O$, and 0.09 μM Fe–EDTA. The plants were treated with 0 (control), 100, 200, 400, and 800 mg L^{-1} COD separately (in the form of sucrose in 10% Hoagland solution), and each treatment was done in triplicate. All plants were treated for 30 days, and the solutions were replaced every 3 days. The stock solution was prepared with 10% Hoagland solution and sucrose. During the experiment, a stock solution was added to each pot twice each day to offset the loss of water from evaporation and transpiration. The physiological changes to the leaves of the plants were observed every 2 days. Plant leaves were rinsed with distilled water and were immediately stored in liquid nitrogen at −80 °C for assay.

2.2. Determination of net photosynthetic rate

The net photosynthetic rate of leaves was analyzed using a portable photosynthesis measurer (Li-6000, Li-COR Inc., USA). Three measurements were carried out per pot.

2.3. Extraction of enzymes

Leaves (500 mg of fresh weight each) were homogenized in 5 mL cold phosphate buffer (0.05 M, pH 7.8) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was used for the enzyme assays.

2.4. Assay of the activity of SOD, CAT, and POD

SOD activity was assayed according to the method described by Beauchamp and Fridovich (1971) with some modifications. The activity was determined by calculating the inhibition of nitro-blue tetrazolium (NBT)'s photochemical reduction. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin, and 50 μL of enzyme extract. The reagents above were prepared with phosphate buffer (0.05 M, pH 7.8) except for riboflavin, which was prepared with deionized water. After mixing, the control reaction mixture was placed in the dark, and the others were illuminated at an intensity of 5000 lx for 15 min. The absorbance was then measured at 560 nm with no illumination of the blank. One unit of SOD activity was defined as the quantity of SOD required to obtain a 50% inhibition of the reduction of NBT. The activity was expressed as units per gram of fresh weight of leaves (FW).

CAT activity was assayed according to the method described by Aebi (1974). H_2O_2 has a maximum light-absorption at 240 nm. CAT can cause hydrogen peroxide to break down, so the reaction solution reduces absorbancy over time. CAT activity can be measured according to the determination of the absorption change rate. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2, and 20–30 μL of enzyme extract. The decrease in absorbance at 240 nm was measured. CAT activity was defined as the absorbance change at 470 nm per minute per g FW using the extinction coefficient $\varepsilon = 0.0394\ cm^2\ mol^{-1}$.

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