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# Effects of treated industrial wastewaters and temperatures on growth and enzymatic activities of duckweed (*Lemna minor* L.)

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ABSTRACT

The efficacy of the removal of contaminants from wastewater depends on physico-chemical properties of pollutants and the efficiency of treatment plant. Sometimes, low amounts of toxic compounds can be still present in the treated sewage. In this work we considered the effects of contaminant residues in treated wastewaters and of temperatures on *Lemna minor* L. Treated effluent waters were collected, analyzed and used as duckweed growth medium. In order to better understand the effects of micropollutants and seasonal variation, the plants were grown under ambient conditions for seven days in summer and winter. Relative growth rate, pigments and phenolic compounds concentrations were determined, as well as the activities of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (G-POD) and polyphenol oxidase (PPO).

The pollutant concentrations varied in the two seasons, depending on the industrial and municipal activities and efficiency of treatments. Treated waters contained heavy metals, nitrogenous and phosphorus compounds, surfactants and hydrocarbons. Compared to the control, duckweed growth of treated plants decreased by 25% in summer, while in the winter due to the lower temperatures and the presence of pollutants was completely impeded. The amounts of photosynthetic pigments of treated plants were not significantly affected in the summer, while they were higher than the control in the winter when the effluent had a high nitrogen amount. High CAT activity was registered in both seasons. Treated plants had significantly lower APX activity in the summer (53%) and winter (59%) respect to the controls. The observed inhibition of the peroxidase activities in the exposed plants, confirms the controversy existing in the literature about the variability of enzymatic response in stress condition.

## 1. Introduction

The discharge of contaminated waters into water bodies can have an important impact on aquatic ecosystems, being a tremendous hazard to both natural ecosystems and human health (Calderón-Preciado et al., 2011; Petrovic et al., 2011); therefore, the wastewaters have to undergo to treatment before being discharged. These treatments are not always designed and operated to eliminate completely the pollutants, thus some residues, consisting of a vast and expanding array of anthropogenic, as well as natural substances, can be still present in the treated waters. The presence of these residues can endanger the habitats due to the toxicity of contaminants, that can disrupt many cellular functions impacting on plant growth (Kohen and Nyska, 2002; Forni, 2014). A better understanding of the effects of micropollutants on not target organisms, living in the receiving water bodies, is important to determine the possible future impacts of treated wastewaters on the ecosystems and for the improvement of wastewater treatment processes.

Lemnaceae are the world's smallest and fast growing angiosperms (Ziegler et al., 2015), representing suitable producers of large amount of biomass. They have a great economic potential and many practical applications in biotechnological and ecological fields (Cascone et al., 2004; Ippolito et al., 2007; Forni et al., 2012; Tel-Or and Forni, 2011; Neagu et al., 2014; Cui and Cheng, 2015). Their morphological and physiological characteristics are favorable features that allow valuable bioassays under limited spatial condition in short time, thus representing model laboratory organisms (reviewed by Forni and Tommasi, 2016). *Lemna* species are used as bioindicators for *in situ* and *ex situ* ecotoxicological assays (Scherr et al., 2008; Forni, 2014; Brain and Solomon, 2007). In these assays growth inhibition, pigments

*Abbreviations:* APX, ascorbate peroxidase; CAT, catalase; f.w., fresh weight; GI, growth index; G-POD, guaiacol peroxidase; HM, heavy metals; PPO, polyphenol oxidase; RGR, Relative Growth Rate; ROS, reactive oxygen species

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content are the most common endpoints considered.

In the literature the oxidative stress, induced by stressors like pollutants and high temperatures, is considered an important subject both for terrestrial and aquatic toxicology and for determining plant tolerance (Apel and Hirt, 2004; Valavanidis et al., 2006; Radić et al., 2011). Plant exposure to environmental stressful conditions can produce the unbalance between reactive oxygen species (ROS) production and scavenging, that causes oxidative stress. In plants, cell molecular and physiological responses are elicited to counteract the oxidative stress induced by ROS overproduction, these include antioxidant defenses (enzymatic and non enzymatic).

Since the presence of even low load of pollutants in treated waters may represent an environmental risk, in this work the complex effluent samples from industrial wastewater treatments were analyzed, and these waters were used as duckweed growth medium. Thus, to determine the extent to which *L. minor* can withstand the presence of multipollutants in natural conditions, the responses of duckweed grown in treated wastewaters were analyzed with reference to changes of pollutant load and seasonal temperatures. We have considered the following parameters: (1) pollutant load of wastewaters; (2) plant growth rates; (3) contents of chlorophylls, carotenoids and phenolic compounds; and (4) the activities of antioxidant enzymes, such as ascorbate peroxidase (APX) and guaiacol peroxidase (G-POD), catalase (CAT) and polyphenol oxidase (PPO).

## 2. Materials and methods

### 2.1. Plant growth conditions

*Lemna minor* plants were collected in a pond in the Latium Region (Italy), duckweed was maintained as stock cultures and prior the experiments, plants were acclimated in Hoagland medium, pH 6.5 (Forni et al., 2001).

Depending on possible variation of the effluents over time, due to the industrial activities and performance of wastewater treatment plants, we decided to sample the effluent, from Industrial District of Latium Region, in two different period, e.g. summer and winter.

To keep the conditions as close as possible to the natural ones, the experiments were performed using wastewater samples “as is” (Wang, 1990). The experiments were undertaken indoor under environmental conditions in summer (July) and in winter (December). During the summer the registered ambient temperatures ranged between 28 °C (min.) and 38 °C (max.). In the winter the minimum of temperature was 18 °C and maximum was 20 °C.

For the experiments, 4 g/L duckweed plants, corresponding to 80% surface coverage, were rinsed and inoculated in plastic bowls (20 cm in diameter), containing 900 mL of 1/10 strength Hoagland medium, pH 6.5 (controls) (Forni et al., 2001) or 900 mL treated wastewaters (treated). Evapotranspiration rate was also considered. Each treatment was performed in triplicate and a randomised block design was applied to the experiments.

Plants were grown over a period of a week, when the consequent potential growth inhibition and other effects can be estimated. At the end of the experiments the plants were sampled, rinsed, gently blotted with paper and frozen at −80 °C until the analyses reported below.

All chemicals were purchased from Sigma, unless otherwise stated.

### 2.2. Growth and pigments concentration of the duckweed

After 7 days of treatment, plants were collected, surface-dried by gentle blotting between layers of paper towels and fresh weight was detected. Growth was determined as Relative Growth Rate (RGR, g $d^{-1}$) as follows: RGR = log N final fresh weight (g.) – log N initial fresh weight (g.)/ time (d).

Concentrations of photosynthetic pigments were determined in plant samples (200 mg f.w.) homogenized in methanol. Extracts were incubated in the dark and shaken (100 rpm, New Brunswick, Orbital Shaker, USA) for 1 h. Supernatants were collected from centrifuged samples and their absorbances at 665 nm (Chl a), 652.4 nm (Chl b) and 470 nm (carotenoids) were determined by spectrophotometer (Cary 50 Bio UV–Visible Spectrophotometer). Pigments concentration was determined according to Lichtenthaler (1987). Results are expressed as milligrams of chlorophylls or carotenoids per gram of plant fresh weight. The data are expressed as mean values ± ES.

### 2.3. Extraction and quantification of phenolic compounds

200 mg of plants were homogenized in liquid nitrogen (LN) and suspended in 4 mL 0.1 N HCl (Legrand, 1977). Samples were incubated and shaken (100 rpm, New Brunswick, Orbital Shaker, USA) at 5 °C for 1 h, and then centrifuged. Supernatants were collected and pellets resuspended in 4 mL di 0.1 N HCl and then centrifuged again. Phenols amount was determined in the two pooled supernatants by Folin Ciocalteu method (Booker and Miller, 1998), using chlorogenic acid (CA) as standard, for which a calibration curves was carried out with different concentration solutions of this compound ($R^2$ = 0.999, y = 0.0031x + 0.011, calibration curve of July; $R^2$ = 0.998, y = 0.0021x – 0.014, calibration curve of December). Results are expressed as milligrams of CA equivalents per g of plant fresh weight (Forni et al., 2012). The data are expressed as mean values ± ES.

### 2.4. Determination of enzymatic activities and total soluble proteins

All enzymatic activities were determined in both treated and untreated plants at the end of the experiments. Protein concentration was determined by Bradford protein assay (Bradford, 1976), using bovine serum albumin as standard.

#### 2.4.1. Ascorbate peroxidase (APX, E.C. 1.11.1.11)

The crude enzyme was extracted basing on the method of Nakano and Asada (1981). 250 mg f. w. were homogenized in 2 mL extraction buffer (Corsi et al., 2015). The reaction buffer (1.5 mL total volume) was composed by 250 µl of the extract, 1 mM EDTA, 0.5 mM ascorbic acid and 1 mM $H_2O_2$. The decrease in absorbance was followed at 290 nm for 300 s by a spectrophotometer (model Cary 50 Bio UV–Visible Spectrophotometer, Varian, Netherlands). One enzyme unit (EU) is the amount of enzyme that oxidized 1 µmol of ascorbate per minute at test condition (Corsi et al., 2015). The results were expressed as EU per microgramme of proteins.

#### 2.4.2. Catalase (CAT, EC 1.11.1.6)

The enzyme was extracted and its activity detected according to the method of Aebi (1984), modified as follows. 250 mg f.w. were homogenized on ice and suspended in 3 mL extraction buffer, containing 0.1 M phosphate buffer pH 7.3, 3 mM EDTA and 1% (v/v) Triton X-100. The extracts were centrifuged and 75 µl, of the supernatant was added to reaction buffer containing 0.1 M phosphate buffer, pH 7.3, 3 mM EDTA, 10 mM $H_2O_2$.

The activity was determined spectrophotometrically at 240 nm (model Cary 50 Bio UV–Visible Spectrophotometer, Varian, Netherlands). The activity is expressed as µmol/mL of $H_2O_2$ consumed /minute, extinction coefficient ε = 0.036 $mM^{-1}$ $cm^{-1}$.

#### 2.4.3. Guaiacol peroxidase (G-POD, EC 1.11.1.7) and Polyphenol oxidase (PPO, EC 1.14.18.1)

250 mg (f.w.) of samples were homogenized in LN and suspended in 2 mL extraction buffer containing 50 mM phosphate buffer pH 6.5, 0.4 mg $MgCl_2$ $6H_2O$, 100 mM EDTA, 20 mg polyvinil pirrolidone (PVPP) and 6% (v/v) Triton X-100 (Ayaz et al., 2008). The extracts were kept at 5 °C for 1 h. and shaken at 100 rpm. Then the extracts were centrifuged and the supernatants were collected.

To determine G-POD activity, three millilitres of reaction mixture