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THE ROLE AND INFLUENCE OF ABIOTIC FACTORS IN *LEMNA MINOR* GROWTH AND PRODUCTIVITY UNDER EXPERIMENTAL LABORATORY CONDITIONS

Valentin Jujea [1], Cristian Emilian Pop [1,2], Cosmin Alexandru Munteanu [1,2], Cătălin Dinu [2], Denis Dulubei [2], Adelina Dumitrescu [2], Anca Vârcolici [2], Dorin Lucian Hanganu [1], Nicolai Crăciun [1,2*]

[1] Ecological Society Aquaterra
[2] Faculty of Biology, University of Bucharest, Romania

Abstract
This study examines the influence of abiotic factors over Lemna minor, along with its symbiotic organisms, in its growth rate under laboratory conditions. We explored the ability of the plant to purify water taken from fish ponds under variable conditions (such as temperature, symbiotic organisms, oxygen, nitrites, nitrates and ammonia). The viable specimens were exposed to simulated conditions during 18 months and samples have been taken periodically.
An increased Malondialdehyde (MDA), catalase and polyphenol levels in L. minor indicated reactive oxygen species (ROS) accumulation and oxidative damage has occurred. This is due to ROS growth potential to induce oxidative stress contributing to lipid peroxidation and membrane damage, MDA level was considered as an indicator of the lesion.

Keywords: *Abiotic factors, Lemna minor, oxidative stress*

1. INTRODUCTION

Aquaculture is the efficient meat production sector offering ample business opportunities. In this circumstance, it has grown at an impressive rate over the past decades and it has helped to produce more food fish, kept the overall price of fish down, and made fish and seafood more accessible to consumers around the world. (Fish to 2030, World Bank Report Number 83177). Although extensive methods of aquaculture involving the farming of different commercial fishes in a natural habitat with little inputs and no supplementary feeding has minimum impact on the environment however, the semi-intensive and intensive culture practices involving the usage of more inputs, mostly of high-quality artificial feeds and are lead to the production of large quantities of solid and nutrient wastes into the environment chemicals (Arvanitoyannis and Kassaveti, 2008). Thus, these technologies are detrimental to the environment by causing eutrophication and destruction of natural sites of natural aquatic fauna, decreased biodiversity of natural fish populations by escape of nonnative fish species (Chatlaet. al., 2018, Srithongouthai and Tada, 2017; Rijn, 2013). Also, aquaculture effluents are believed to be associated with other compounds with toxically effects (for example: different pathogens, heavy metals, hormones or antibiotics) who generate a potential risk for human health (Madariaga and Marin, 2016; Windi et al., 2016) andsoil pollution and surface waters by effluent discharge (Boyd, 2003).

*Corresponding author, E-mail address: craciun.nicolae@yahoo.com

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In this context water quality control and waste management are among the most critical stepsand recirculating aquaculture systems (in which water is recirculated between the culture and water treatment stages), has been developed to provide an answer to some of the above mentioned problems since they enable fish production in relative isolation from the surrounding environment.
The use of aquatic macrophytes, such as duckweedin wastewater treatment has attracted global attention in recent years (Popa et al., 2017). Duckweed species have shown characteristics that make duckweed-based systems very attractive, not only for wastewater treatment but also for nutrient recovery. The reason for this is the rapid multiplication of duck-weeds and the high protein content of its biomass which is about 30-49% of dry weight (Oron et al., 1984; Skillcorn et al. 1993).
In addition, like other plants, duckweed species possess biopharmaceuticals with antimicrobial activities (Li et al., 2018; Nicu et al., 2018).
An interest is currently manifested in Lemnacea species (137 known species worldwide) as they proved a high potential in metabolizing waste such as: heavy metals, pesticides, antibiotics, pathogens and massive organic wastes, together with being a novel bioenergy crop with significant potential to accumulate starch. The studied model is *Lemna minor*, one of the most common species of Lemnacea in Europe that has also spread to other continents. Plants are 1-8cm in length and 0.6 up to 5 mm wide with the flower having one egg with two stamens (Khoei et al., 2018). This specie is tolerant tomoderate temperatures (growing at temperatures between 15-33ºC and only decreasing in growth and productivity at 33-36ºC). Its provedresilience to temperature and to other abiotic and biotic factors made it the perfect candidate for the purification of high ammonia waste waters from swine and fish farms,beingalso successfully used to treat and purify city and industrial waters, such as lead-contaminated waters of Devils Lake, North Dakota, U.S.A. (Chang, 2002).
The purpose of this work is to demonstrate the biochemical stress adaptation to different abiotic factors (oxygen level and growth temperature) on *Lemna minor* in a controlled environment semidefinite and indefinite growth media.

2. MATERIALS AND METHODS

The plants and its symbiotic microorganisms used in this study were obtained from Vacaresti Natural Park, fishpond water was obtained from Frasin research station. Aquaculture system of *Lemna minor* took place in a vessel, in static conditions, equipped with air pumps (Aquael OXY BOOST-APR 300, RESUN AC9600, RESUN SP800) under pH, oxygen, temperature and nitrites monitorization (Portable Meter HQ40d).
The two liquid growth conditions were realized using a indefinite medium (Kittiwongwattana and Vuttipongchaikij, 2013). Table 1 shows the characteristics of the waste water used in the coculture sturgeon and salmon, used water in the experiment (analyzed by SGA Suceava). The measured light intensity was 157 lm (lumens).
The temperature of the water tank was set at first for 22^{o}C and in the second stage at 28^{o}C. Oxygenation was kept at a constant of 273 mg/min,samples have been taken periodically, both of water and of vegetal material, in order to check the biochemical parameters.
During the 18 months study period the aquaculturewasmaintained under constant standards in 5 water tanks. Illumination was kept concomitant with the natural circadianrhythmusingthreeneon lamps with full visible spectrum.
Since the water surface was in contact with the air, water had to be added to maintain the constant level and prevent losses due to evaporation.

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Tabel 1. Physico-chemical indicatorsof sturgeon and salmon used water

Nr. crt.	**Wastewater Indicators**	**UM**	**Determined values**
1.	Ammonium	mgN/L	0.076
2.	Total nitrogen	mgN/L	1.5
3.	Nitrates	mgN/L	1.34
4.	Nitrites	mgN/L	0.01
5.	CCO-Mn	mgO_2/L	12.3
6.	Chloride	mg/L	6.0
7.	Conductivity	µs/cm	295
8.	Hardness	^0d	10.3
9.	Iron	mg/L	0.292
10.	Phosphate	mg P/L	0.028
11.	Total phosphorus	mg P/L	0.042
12.	Hydrogen sulfide	mg/L	0.064
13.	Dissolved oxygen	mgO_2/L	11.79
14.	pH	Unit. pH	7.34
15.	Fixed residue	mg/L	192
16.	Tone	mg/L	20.3
17.	Suspensions	mg/L	11
18.	CBO5	mgO_2/L	4.39

Tabel 2. Fishpond water composition

Nr. crt.	Indicators	UM	Determined values, sturgeon water
1	Ammonium	mgN/L	0.053
2	Total Nitrogen	mgN/L	3.23
3	Nitrates	mgN/L	2.733
4	Nitrites	mgN/L	0.0014
5	CCO-Mn	mgO2/L	3.65
6	Chloride	mg/L	1.55
7	Conductivity	µs/cm	322
8	Hardness	0d	13.2
9	Iron	mg/L	0.684
10	Phosphate	mg P/L	0.011
11	Total Phosphorus	mg P/L	0.025
12	Hydrogen sulfide	mg/L	0.096
13	Dissolved oxygen	mgO2/L	10.83
14	pH	Unit. pH	8.10
15	Fixed residue	mg/L	220
16	Sulfates	mg/L	33.4
17	Suspensions	mg/L	67

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Table 3. Oxygen content, temperature and light intensity in this experiment

Temperature (ºC)	Oxygen (%)	Light intensity (lm)
22	21	157
28	21	157

Lemna minor was seeded in 60% proportions in each watertank (table 4):

Table 4. The proportion of chlorophyll for Lemna minor at different tipe of water and temperatures

	L. minor in the waste water				*L. minor* in an undefined environment			
Growthconditions (temperature/aeration)	Chlorophyll (mg)	Chlorophyll b (mg)	Total Chlorophyll (mg)	Chlorophyll a/ Chlorophyll b (mg)	Chlorophyll a (mg)	Chlorophyll b (mg)	Total Chlorophyll (mg)	Chlorophyll a/ Chlorophyll b (mg)
22ºC	147.1	49.9	197	2.947896	54.6	21	75.6	2.6
28ºC	149.1	52	201.1	2.867308	63	24.5	87.5	2.571429
22ºC aerare 3 l/min	138.5	45.3	183.8	3.057395	48.5	18	66.5	2.694444
28ºC aerare 1,5l/min	143.2	47.8	191	2.995816	51.2	19.5	70.7	2.625641

Samples of water and viable specimens have been taken periodically and recorded. Water analysis was performed to detect the levelsof pH, nutrients and toxic gases (CO_2, ammonia, nitrates, hydrogen sulfide) absorbed by the Lemnacea populations and chemical parameters of used water have been detected (Ammonia, Azotate, Phosphorus).

Water samples were put on ice and centrifuged at 10000 rpm, the supernatant was harvested and analyzed for ammonium ion (NH_4^+), nitrite ion (NO_2^-) and nitrate ion (NO_3^-).

To determine the chlorophyll content we used the protocol described by Porra et al. (1989) and Harris & Baulcombe (2015), the amount of chlorophyll / mg of fresh vegetable tissue is depending on the amount of plant material used (in this case we used 50 mg of vegetal material).

The determination of catalase activity, peroxidase activity, lipid peroxidation (MDA) level and total polyphenols were also established.

Evaluation of the catalytic activity involved weighing about 100 mg of fresh vegetable material that was homogenized in 0.1 M sodium phosphate buffer, pH=7 in a ratio of 1:20 (w / v) and after 15 min. of cold incubation (4°C) was centrifuged at 4°C for 10 min. at 10000 rpm (Maehly & Chance, 1967).Catalase activity was determined by the decrease in absorption at 240 nm due to the consumption of H_2O_2 (Aebi, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 100 µL of plant extract. The total volume of the reaction mixture was 2 ml. An activity unit was defined as µmol of H_2O-oxidized min-1. The specific enzymatic activity of catalase was expressed as units/g of fresh plant material (Subhadra et al., 1991; Varga et al., 2013).

Lipid peroxidation has a significant impact over the plasma membrane structure and permeability. It was indirectly estimated as a result of malondialdehyde (MDA), a by-product of lipid peroxidation that reacts with thiobarbituric acid (TBA) (Varma and Dubey, 2003). Thus, about 200 mg of plant material was homogenized with 1 ml of 0.5% thiobarbituric acid in 20% trichloroacetic acid (w/v). The homogenates were then incubated at 100°C for 30 min and the reaction was stopped by

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immersion in an ice bath. The samples were then centrifuged at 10000 rpm at 4°C for 10 min and the absorption was measured at two wavelengths, 532 and 600 nm. The amount of MDA was calculated using a molar extinction coefficient equal to 155 mmol L-1 cm-1 and expressed in nmol MDA/g of fresh material (Varga et al., 2013).

Last, but not least, to determine the total polyphenols content, we used the procedure described by Slinkard and Singleton (1977) using the FolinCiocalteu reagent that uses gallic acid as the standard. Thus, 50 mg of fresh plant was treated with acidified methanol with HCl (0.1%) in a ratio of 1:20 (w / v). Then, the plant tissue was placed on an ultrasonic bath for 30 minutes. taking care that the temperature does not exceed 20ºC. After that, 1 ml of alcoholic extract was treated with 0.5 ml of FolinCiocalteu reagent and vigorously stirred. After 3 minutes, 1.5 mL of Na_2CO_3 (2%) was added and the mixture was incubated at room temperature for 2 hours with intermittent agitation. Absorbance was then measured at 760 nm in a spectrophotometer. Amounts of total phenolic compounds from *Lemna minor* were determined as gallic acid equivalents (GAE) using a gallic acid curve, the equation that was obtained from a standard graph of gallic acid (Gülçin et al., 2010).

3. RESULTS AND DISCUSSIONS

The principle of waste water treatment with Lemnaceae sp. is based on its ability to purify wastewater in conjunction with aerobic and anaerobic bacteria. Usually, the surface water microcosmos can be split in three different areas. These areas are the aerobic area, the anoxic area and the anaerobic area (Skillicorn et al., 1993). In the aerobic area, organic materials are oxidized by aerobic bacteria using atmospheric oxygen transferred by the *Lemna minor* roots (Tchobanoglous and Burton, 1991). Nitrification, being a process of oxidizing ammonia and ammonium ions resulting in nitrites and nitrates, and denitrification occurs in anoxic zones. There, organic nitrogen is decomposed by anoxic M.O. into ammonium and orthophosphate, which are intermediate products used as nutrients by Lemnaceae sp. (Smith and Moelyowati, 1998).

Selvarani et al. (2015) showed that *Lemna minor* achieved a maximum efficiency of 96%, 98%, 98%, 96%, 79% and respectively of 79% of removing NH_3, NO_2, NO_3, PO_4, BOD and COD from municipal wastewater. Through this investigation, it was concluded that elimination of nutrients from waste water can be reduced by treatment with *L. minor*, and therefore the process of eutrophication of water can be avoided.

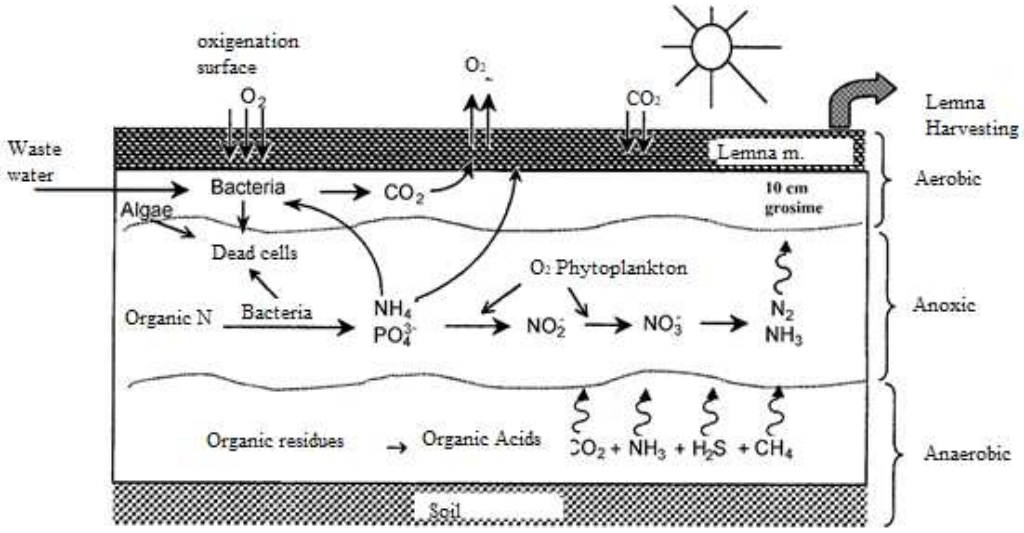

Figure 1 Schematics of biological processes that develop in waste water treatment using Lemna m. (Smith and Moelyowati, 2001)

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Due to the absence of motility, plants have elaborated their own survival mechanisms in order to adapt to changes in the environment. Although many of the mechanism of which plants perceive these changes are unknown, however there is a good understanding of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) being the central components ofsignal transduction in this process.

ROS plays a keen role in structural alteration and cellular signaling, activating defense responses to abiotic and biotic stress (Levine et al., 1994; Prasad et al., 1994; Alvarezet al., 1998; Allan& Fluhr, 1997).

H_2O_2, due to its feed-back mechanism and long half life (hours to days) together with its high diffusion rate (it can travel through membranes easily) is considered to be a signal molecule in ROS signaling and it is controled by catalase (CAT). Catalase is an enzyme that degrades H_2O_2 in water and oxygen, thus being one of the main antioxidizing enzymes, as one of the main factors that maintain redox homeostasis. CAT removes H_2O_2 generated during mitochondrial electron transport chain during β-oxidation of fatty acids and during oxidative photorespiration (Scandalios et al., 1997).

Tabel 5. L. minor catalase activity in used water at 22 and 28 degree celsius with and without aeration

L. minor in sturgeon water		
Growth conditions/temperature/aerification. Catalytic activity (U/mg wet substance)		
temperature	oxygen (mg)	U/mg
22°C	9.37	42.27 ± 0.61
28°C	9.37	45.57 ± 0.33
22°C + aerification 3 l/min	9.37	52.65± 0.28
28°C + aeration 1,5l/min	9.37	52.37 ± 0.28

Lipid hydroperoxides are exceptionally stable under favourable conditions, such as low temperature, dilute solution, the presence of antioxidants and the absence of catalysts such as iron salts (Gardner, 1987). The determination of LHPOs in photosynthetic tissues is potentially more problematical as light absorption by plants other than lipids can initiate the formation of free radicals in sintetized photooxidation (Chan and Cotton, 1987). Photosynthetic tissues may contain higher levels of LHPO than non-photosynthetic tyissues resulting from reactive oxygen species-damage during the extraction process. It is important to relate the hydroperoxide content to the total lipid content of tissue.

Tabel 6. Level of lipid peroxids under experimental conditions described above

L. minor in synthetic environment	
Growth conditions/temperature/aerification MDA (nmols/g fresh tissue)	
Temperature	nmols/g fresh tissue
22°C	3
28°C	3.2
22°C + aerification 3 l/min	5
28°C + aerification 1,5 l/min	3.6

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The enhancement of phenolics concentrations in stressed plants can improve their antioxidant capacities, since in such systems, phytophenolics can act as antioxidants by donating electrons to guaiacol-type peroxidases for the detoxification of H_2O_2 produced under stress (Sakihama et al., 2002). Under conditions of severe stress it has been proposed that phytophenolics, particularly polyphenols, function as antioxidants to support the primary ascorbate-dependent detoxification system as a backup defense mechanism of vascular plants (Sakihama et al., 2002; Mihai et al., 2011).

Tabel 7 .Concentration of polyphenols under experimental conditions described above.

L. minor in synthetic environment	
Growth conditions/temperature/aerification total polyphenols (µg/ mg extract)	
Temperature	µg/ mg extract
22°C	22.0 ± 0.8
22°C + aerification 3 l/min	22.0 ± 0.11
28°C	24.0 ± 0.9
28°C + aerification 1,5 l/min	19.0 ± 0.10

The responses of *Lemna* and *Spirodela* photosynthesis to temperature are not typical though of C_3 plants. For example, in *Helianthus annuus* L., sunflower, a C_3 plant, a temperature optimum of 20°C was observed at both low (300 pE m -2 s -1) and high (1800 pE m -2 s -z) light intensities, and temperature increasing above 20°C decreased photosynthesis (Hew et al., 1969a, b), whereas in our experiments temperatures above 20°C did not decrease photosynthesis. Ashby and Oxley (1935) also found for *Lemna* that higher temperatures did not depress CO_2 assimilation rates, with CO_2 assimilation being rather constant from 18-29°C.

More significantly, the duckweeds do not follow the usual pattern of a C_3 plant's photosynthetic response to temperature and light, that is, temperature and light optima are usually similar to the conditions at which the plants are grown (Chollet and Ogren, 1975). The duckweeds instead seem to have the higher temperature optima characteristic of C_4 plants. For example, Zea mays L., maize, a C_4 plant, showed a photosynthetic optimum at 30°C, with rates at thistemperature almost double those observed at 20°C at a light intensity of 6000-10000 ft-candles (Moss, 1963). The duckweeds had h temperature optimum in the 30-35°C range with lower photosynthesis at 20°C, although the difference in photosynthesis between 20 and 30°C was much less pronounced than for the C_4 plant. Based on our experiments and those of other investigators (Ashby and Oxley, 1935; Bauer et al., 1976) the duckweeds appear to be C_3 plants. Their high temperature optima and light intensities for saturation of photosynthesis make them atypical C_3 plants though, worthy of further study.

Tabel 8.Concentration of chlorophyl and the raport between clorophyl aand clorophyl b inLemna minor. Results are expressed inµg/g wet substance.

Growth conditions (temperature/illumination)	Chlorophyll a (mg)	Chlorophyll b (mg)	Total Chlorophyll (mg)	Chlorophyll a (mg) Chlorophyll b (mg)
22°C	49.03333	16.63333	65.66667	2.947896
28°C	52.31579	17.33333	69.64912	3.018219

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Generally, chlorophyll breakdown is a common event during senescence and it is assumed that chl loss is linked to protein degradation (Hashimoto et al., 1989). An increased rate of senescence and activity of different proteolytic enzymes was noticed in wheat and rice exposed to osmotic stress (Srivalli et al., 1998). In our study, temperature and oxygen excess may have promoted premature senescence also because that osmotic agent caused not only the degradation of chl but also of protein content in duckweed plants. It is noteworthy that massive proline accumulation observed under oxygen and temperature -induced stress could not ameliorate consequent oxidative damage and inhibitory effects on L. minor growth, though it might have contributed to more efficient antioxidant enzyme activity (Hayat et al., 2012). Like other plants, L. minor has a high adaptative capacity (Pascale et al., 2016).

4. CONCLUSIONS

Small aeration periods, together with moderate temperature rises that amplifies the biodegradation process show an improvement in the epuration processes of *Lemna minor*. The analysis of biochemical markers (chlorophyll content, catalytic activity, lipid peroxides and polyphenol levels) confirms the plants ability to eliminate abiotic pollutants from the culture media and, furthermore, anundefined chemical environment seems to protect the plantfrom chemical and biochemical oxidative reactions that normally occur and lead to oxidative stress.

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*Corresponding author, E-mail address: craciun.nicolae@yahoo.com

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*Corresponding author, E-mail address: craciun.nicolae@yahoo.com