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3. Experimental research compared aquaculture of certain species of the *Lemna genus* with demonstration of environmental requirements and of the adaptations to environmental conditions specific to aquatic eutroph-polytroph ecosystems

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Abstract: The macrophyte plants Lemnaceae are abundant in the tropical and subtropical regions but now present in the Danube Delta. The plant grows in water with high levels of N, P, and K, synthesizing protein at a remarkable rate. Used as food and feed in some regions it is currently attracting interest especially for its ability to metabolize and purify wastewater. Our culture technique of duckweed was standardized in water tanks and a series of analytical methods for determining the chemical parameters of waste water were optimized. Phenotypic biodiversity of the aquatic communities was analyzed using the Biolog Ecoplates system on a total of eight samples, concluding in a symbiosis between the plants and the microbial community that can be linked to Lemnaceae high wastewater purification abilities, that transforms the highly toxic environment into an environment conducive to the development of aerobic organisms.
Keywords: Lemnaceae, bioremediation, biological filtration

INTRODUCTION

In the Romanian flora, there are five species of Lemna (fam. Lemnaceae: *Lemna minor, Lemna gibba, Lemna trisulca, Spyrodella polyrrhiza, Wolffia arrhyza*) and a new species from North America - *Lemna minor*, present only in the Danube Delta. *Lemna minor* was probably introduced unintentionally by commercial ships movement or while it was under the turion stage, during wintertime. Lemna species have been under in-depth studies by several large teams and funded by biologists from several research centers in the US, Denmark, Germany, Japan, who for 25 years have conducted many studies on lipid composition, carbohydrates, proteins, vitamins, proenzymes, etc. It has thus been concluded that Lemnaceae (duckweed) plants have higher protein and lipids content than corn (Appenroth et all, 2013). The US experience regarding this plant has also been applied in the U.E. by developing a patent for the macro-scale refinery that produces better octane fuels than biodiesel extracted from corn or rapeseed. Also, the applied scientific research experience from Denmark brought novelties by making the first foods from *Lemna minor* in 2018-2019. These products are intended to be used mainly on the International Space Station, the duckweed having exactly the ideal biochemical composition required to make complete foods without other types of dietary supplements.

Recommended to be one of the candidates for the future colonization of the planets, where the first human colonies will take place. It would also be important if we were able to find out the efficiency of fermentation and biogas production from *Lemna minor* and *Lemna gibba*. The reproduction Lemna species and the increase in biomass production and productivity occur only if there are no fish or other animals in the growth environments which by feeding reduce the huge exponentially potential of uninterrupted reproduction of duckweed.

The population growth of duckweed is a gaussian type growth pattern similar to that of bacteria. Nevertheless, collecting duckweed mass must be done only when the population reaches the maximum

supportability of the lake luster destinated for the growth of duckweed, such as decantation lakes for tertiary filtration and maturation of the filtering water.
The water in question becomes generally cleaner than the water in the river reef basin (stream, river) or the lake water, adjacent ponds with extensive wetlands in the Danube Delta or the large Danube ponds. Lemna species become the best solutions for purifying already heavily polluted lakes (lakes in Latin American countries - Venezuela, San Salvador, Honduras, Mexic - where fish have already disappeared).

The purification of these large lakes polluted extremely with oil residues, where all the fish species died long before, first became lakes of anaerobic fermentative microorganisms (arhebacteria) and in the last two years, they became possible living conditions for exponential growth of *Lemna minima* plants.
Lemna species restore these compromised lakes in a much shorter time than previously thought. Proof are the photos taken by NASA's geostationary satellites that have been taking images for several years showing how the growth of duckweed (under anaerobic and fish-free lakes) has increased production to over 200 tonnes /ha/30 days. Water purification is very advanced. Other studies also show the ability to detoxify and metabolize heavy metals (Cu, Cd, Zn, Pb) as well as antibiotics, which is why they are very effective in fish farms and facilitate the biochemical protection of filtering microorganisms because all types of feeds and medicines used in aquaculture also contain sufficient antibiotics to inhibit the normal development of filter microorganisms in recirculating aquaculture systems (RAS).
These highly aerobic oxyphilic bacteria are very sensitive to antibiotics, which is why the extremely beneficial effect of Lemnaceae plants makes them capable of advanced detoxification (even when metabolized in excess ammonia conditions above the limits of all higher plants). The energy resulting from the photosynthesis processes may be very efficient (more efficient than in most higher plant species) (Kwan & Smith 1988).

Due to the fact that these plants float and live on the surface of the waters, where they are exposed to the largest possible sun exposer on the planet, it is possible that they may have developed over time through the process of evolution and adaptation, biochemical mechanisms of sun protection for chlorophyll, chloroplasts or other specific cytoplasmic reactants that are involved in all complex metabolic processes. It is already certain (and we also performed experiments by demonstrating at what concentrations of ammonia *Lemna minor* plants grow (experiment performed by the end of 2018). At these concentrations all the other phanerogams and even cyanobacteria died but yet Lemnaceae plants not only survive but also thrive, metabolizing ammonia successfully. Thus, it transforms the highly toxic environment into an environment conducive to the development of aerobic organisms (higher plants, algae, protists, invertebrate animals and aquatic vertebrates) resuming the biogeochemical or biocenotic cycles in the aquatic ecosystems of lakes (lakes, puddles and artificial lakes) (William et all., 1978, Lepp, 1981). Our studies have been made in comparison with all the five duckweed species from the Romanian flora. We have based on the extremely high capacity of these species of plants to treat wastewaters altogether with its detoxification abilities.

The high metabolization of antibiotics by the Lemna species makes it possible to purify water but also increases the value of the plants for this purpose - the purification of antibiotics that destroy in the natural ecosystems the total ecological balance by killing the aerobic microorganisms that efficiently recalibrate the biological filtration through RAS (Recirculating aquaculturre system) - *Nitromonas sp., Nitrobacter sp.*(Leng et all, 1995)

MATERIALS AND METHODS

To monitor the efficiency of the Duckweed bioremediation process we have developed a series of analytical methods for determining the chemical parameters of wastewater (ammonium, nitrate, nitrate, phosphorus) by optimized methods. Mineralization was performed by autoclaving the sample in the presence of sodium hypochlorite and alkaline treatment was performed with NaOH.
Thus, (1) the Fenat method of colorimetric determination of ammonium ion from solutions ($NH4 +$) (Koroleff, 1976) was set up; (2) method of determination of nitrate ion (Cataldo et al., 1975), (3) method of spectrophotometric determination of nitrite ion by derivatization with captopril (Porché, 2014), (4) Phosphorus (Fig. 1) dosing by Fiske--Subbarow method).

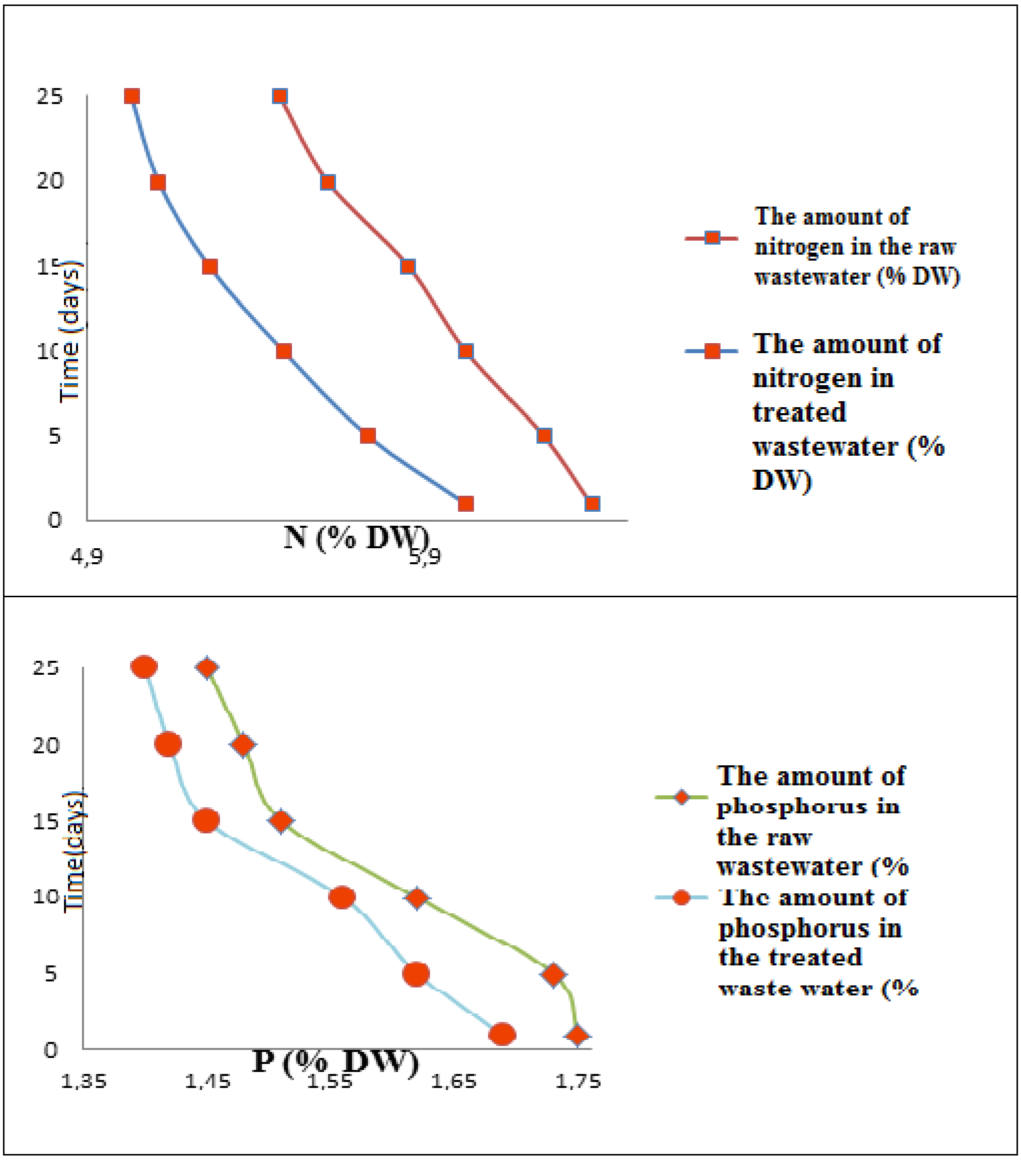

Figure 1 The amount of nitrogen and phosphorus in raw wastewater and in the treated waste water (%)

As can be seen, *Lemna gibba* in treated wastewater media behaves much better than in the case of the raw **wastewater environment at different time intervals.**

Species	Dry quantity (DW) (% dry weight)	N (% DW)	P (% DW)
Lemna gibba	5.29	6.39	1.75
Lemna minor	5.06	6.12	1.40
Spirodela polyrhiza	5.30	6.42	1.82
Lemna trisulca	5.10	6.26	1.53
Wolffia arrhiza	5.08	6.19	1.47

Figure 2. Concentrations of N, P in aquatic plants at pilot scale. Concentrations are given as the means obtained from a harvest of Lemna spp. (October 2017)

For *Lemna minor* crops, under optimum conditions the pH value should be between 6.5-8 and 6-33ºC. Under these conditions, growth is very fast. At 6-7ºC the resistance forms or the plant, which descend to the bottom of the standing waters, are produced. In spring, plant growth and lifting are restarted. Other optimal conditions would be the lack of competition with weak algae and wind, otherwise it causes waves that can overturn the plants.
Another factor in the growth of *Lemna minor* is rainwater. The plant needs an excess of N, P and K, which must be in the concentration of 20-30 mg/l increasing the protein concentration.

Selected methods of water analysis

The methods used to analyze the physical-chemical characteristics of water loaded with known concentrations of nutrients or toxic gases (carbon dioxide, ammonia, nitrites, nitrates, hydrogen sulphide) absorbed by *Lemna minor* and other plant populations and the chemical parameters of the wastewater (Ammonium, Nitrate, Phosphorus) were detected by our adapted methods. The biological and biochemical parameters of the lacustrine lactic aquatic ecosystem can be potential indicators of ecological stress.

EcoPlate Biolog method on the intimate biological filtration mechanisms of the Lemna species, studied comparatively in natural conditions and captive-controlled crops.
The purpose of this research was to characterize the phenotypic biodiversity of aquatic microbial communities associated with aquatic species: *Lemna trisulca, L. minor, Wolffia arrhiza* and *Spyrodella polyrrhiza*.

In this study, a total of 8 samples were harvested and analyzed, including 4 samples of water collected from ponds and 4 samples of aquatic plants represented by *Lemna trisulca, L. minor, Wolffia arrhiza and Spyrodella polyrrhiza*.

The EcoPlates Biolog System was used to study the metabolic responses of microbial communities in 11 samples of water from anthropogenic aquatic ecosystems in Arges County. The results of dynamic macroscopic analyze is correlating with the diversity of microbial strains in the analyzed water samples. On the first day of macroscopic observation by counting and calculating the microplate scores it was noted that most samples exhibit increased microbial biodiversity.

The pattern of use of carbon sources was analyzed based on the determination of the following parameters:- average well-color development (AWCD), a parameter that describes the average use of C sources by microbial communities. It is determined that the mean difference between the absorbance values of the carbon source wells and the values of the control wells $AWCD = \Sigma ODi / 31$ (Garland & Mills, 1991) Parametrul AWCD reflectă capacitatea oxidativă a microorganismelor și poate fi utilizată ca indicator al activității microbiene, o valoarea mare a indicelui indică un număr mare de substraturi C metabolizate.

The obtained results of the AWCD parameter and shown in figure 3, obtained at different time periods (24h, 72h, 96h, 192h and 216h) indicated that the microbial communities in the analyzed aquatic basins show a lower metabolic activity. compared to the metabolic activity of microbial communities associated with aquatic plants. At 24 h, the lowest values of the AWCD parameter were recorded between 0.98 and 2.21. The lowest value in terms of microbial activity was observed for the water samples taken from the water basins of the *Lemna trisulca* and *Spyrodella polyrrhiza* plant species, and the largest water sample from the *Lemna minor* basin. The absorbance values read at 450 nm indicated an increase in the metabolic activity of the microbial populations in the analyzed aquatic basins, at 96-hour time periods. The highest values of metabolic activity were observed at 144 h of incubation for water samples from the aquatic basins of the species of *Lemna* and *Spyrodella polyrrhiza* and at 168 h from incubation for water samples from the aquatic basins of *Wolffia arrhiza*.

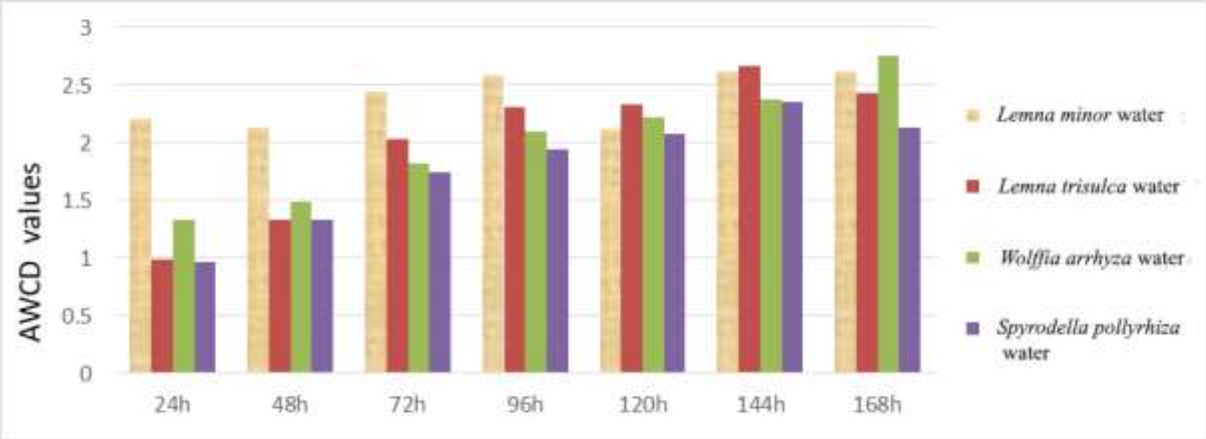

Figure 3. AWCD values for microbial communities in water samples. (in the legend you have to changed apa in water)

Protocol based on persulphate oxidation, required to measure total nitrogen in the water
We have adapted this protocol after the one developed at Berkeley University of California, College of Natural Resources. We have achieved this protocol because the spectrophotometric methods for determining the level of nitrate, nitrite, ammonium and phosphorus ion we have used are only sensitive to inorganic compounds, but there are organic compounds in the wastewater of aquaculture.

Phenat method for the colorimetric determination of ammonium (NH_4^+) in solutions (References: Koroleff, 1976. Determination of ammonia. In Methods of Seawater Analysis (Grasshoft, 1976) The resulting data was used to generate a standard curve against which we can further determine the concentration of ammonium from any solution (wastewater of aquaculture).
Spectrophotometric determination of nitrate (Cataldo et al.,1975) Rapid colorimetric determination of nitrate in plant tissues by nitration of salicylic acid. Commun. Soil Science and Plant Analysis 6 (1) 71-80)
After the samples were brought to room temperature, we measured the absorbance at UV / VIS spectrophotometer at a wavelength of 410 nm against distilled water.
Method of spectrophotometric determination of nitrite ion by derivatization with captopril (Porché, 2014) Spectrophotometric determination of nitrite by derivatization with captopril – Thesis, Miami University, Oxford, Ohio)

RESULTS AND DISCUSSIONS

Species of Lemna were grown in tall pools and illuminated with fluorescent tubes. The intensity of illumination and the distance between the light source and the water level were tested to see the favorable conditions for growing the species of interest.
Several conclusions of the experiments on the growth of Lemna species under laboratory conditions:
In experimental vessels we noticed that the most favorable light for Lemna crops at a water depth of 25-35 cm) is 36 watts. Virtually one neon tube of that power is enough.
If we use a 2-tube lamp, there is already too much light in the filtration system, and green algae and even denser populations of algae and cyanobacteria that compete with the Lemna population are starting to develop and hinder the exuberant development of Lemna crops.
The ideal neon tube distance to water is about 30 cm. Placing them at a smaller distance leads to the development of cyanobacteria.
If we have a permanent recirculation system with a small recirculation pump that does not lead to the *Lemna sp.* plant overturning, the plant growth rate increases.
The length of *Lemna minor* roots is about 3-5 cm in poor crops in organic matter, especially if the aquaculture environment does not have fish. On the recirculating systems of our measured average experiments of *Lemna minor* roots, it reaches 10-12 cm in length, which is even thicker and more vigorous.
Cultivation should be done as much as necessary, but with 90% of the surface area of the aquaculture area occupied with Lemna, in order not to create a dangerous break for algal species, especially cyanobacteria (*Mycrocystis aeruginosa, Nostoc sp*.).
At the same intensity of light, *Lemna gibba* plants develop very well, having the capacity to divide at least 2 times larger than the *Lemna minor*, which we have not found in the specialized literature.

Observing this we concluded that it could be an improvement actor in filtering and recirculating water from RAS from super intensive aquaculture farms.

In some experimental vessels we cultivated *Lemna trisulca*, the only species that lives both on the water surface and in depth. We have experienced the cultivation of this species in 3 experimental variants:

a. on *Ceratophyllum submersus* plants - as we have found them associated in the natural environment of the Gurban Valley - Comana Natural Park.

b. on a rabbit mesh network parallel to the slats frames to be well stretched. These bars are placed parallel to each other. Woven plants as a network develop very well if the water comes with enough organic substance from the aquaculture basins of the fish. The lifecycle must be permanently reophilized by a recirculation pump (brook-type micromedium or slow flow but constant flow filter).

c. High-grain filtering polyurethane foam or special polyurethane foam strands used for retaining as much organic deuterium, especially used in aquaculture recirculating systems.

In all systems used in experiments we used a higher intensity of light (for experimental vessels we used a light intensity of 4 tubes of 18 watts at a water depth of about 30 cm of water, thus provided the plant with harmonious conditions of development according to their ecological requirements. The lateral illumination can be provided by a proposed new type of long-life aquarium with vertical illumination lamps and two lateral lighting systems in such a way that the *Lemna trisulca* filter population grows illuminated from all sides. After filtration the water has to be sterilized with a UV lamp - before it returns to the aquaculture environment.

CONCLUSIONS

AWCD values have shown a higher metabolic activity of microbial communities associated with aquatic plants compared to the metabolic activity of free native microbial communities, or attached to medium support (substrate, vessel walls) in the aquatic basins analyzed.

The metabolic diversity of microbial populations in water samples taken from aquatic tanks was lower than that of the microbial populations associated with aquatic plants, suggesting a greater diversity of microbial communities adhering to the surface of aquatic plants compared to the free suspended water mass.

It is clear and almost certain that we have a very wide variety of associations of photosynthetic microorganisms, aerobic bacteria, aquatic fungi, aerobic/anaerobic microorganisms living in areas with less oxygen, usually under the biodiversity of the roots and the lower part of the leaves which do not have direct contact with water and dissolved air.

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