HOW TO MAINTAIN PHOSPHORUS LEVEL IN SOIL AND GROUNDWATER BY USING MICROBIAL PHYTASE AS A FEED ADDITIVES

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ABSTRACT

The changes and the ongoing developments that the Albanian livestock sector has experienced are naturally associated with the expansion and growth of pork production. In the pig sector, today we can discuss contemporary technology, full balanced nutrition, specialized breeding and qualified management. One of the most common causes of water and soil pollution in the areas with high concentration of agricultural and livestock farms are agricultural technologies and raw animal waste that is scattered everywhere. Excessive animal manure and fertilizer inputs do cause various environmental problems, related to the accumulation and elevated leaching, runoff of nutrients (N and P) and heavy metal to ground water and surface water. The P-excretion on the pigs and poultry faeces is potential source for soil and water pollution, due to his high level on the subsoil water and destruction of the ecosystem. The present paper tests the effects of the microbial phytase (Aspergillus niger, NATUPHOS) 750 FTU/kg on the increase of P availability and animal performance, as well as in the reducing of environmental pollution. Thirty-six piglets (Large White x Landras) of four litters were transferred to flat-decks and allocated to 2 groups (A and B) with 18 animals (9 males and 9 females), respectively. The control group (A) was feed with a balanced diet, containing mono calcium phosphate. The experimental group (B) was feed with low level of phosphorus, without inorganic phosphorus. This group was supplemented with NATUPHOS phytase 750 FTU/kg feed. During the six weeks of experimental period, Body Weight (BW), Daily Weight Gain (DWG) and Feed Conversion Ratio (FCR, kg feed/kg Body Weight Gain were measured weekly. Data are presented as arithmetic means with standard deviation of the mean (Mean ± SD). The supplementation of microbial phytase preparation (Aspergillusniger, NATUPHOS) 750 FTU/kg feed was reduced the P-excretion. The P-excretion was

reduced by 20-25%, provided that pig's diets can be supplemented with an economical and efficacious level of phytase that will allow all of the supplemental inorganic P to be removed from the diet (Cromwell and Coffey 1991).
Keywords: phosphorous, groundwater, microbial phytase, feed additives

1. INTRODUCTION

In Albania the transition by collective to private agriculture has been conducted with sufficient negative phenomena on the land use and management of natural resources (Lushaj and Suljoti 2005). During the last period some significant changes are considered for the Albanian livestock development. But this sector continued to be more problematic as well on the development level as on environment respect. Year after year, the number of livestock farms is growing, but the size of livestock farms is just small.

The changes in environmental performance have been closely linked with the efficient energy use; reform of municipal utilities through such measures as eliminating subsidies and raising tariffs; establishment of basic environmental protection and management systems; improvement of natural resource management and public participation in environmental issues.

Albania continues to application the reforms and developed good institutional and regulatory capacities for managing environmental issues. The roles of the public and private sectors need to be considered according the pollution intensity (solid pollution, the potential collapse of water, water contamination from agricultural or industrial pollutants; energy inefficiency; threats to natural resources; etc.). A regulatory framework and institutional capacity for environmental management needs to be installed or to be applied. Here, the access of rural populations is very limited or inexistent (Bregasi and Veizaj 2007).

Nowadays, pollution is a serious threat to all parts of our environment, including the soil, ground water and surface water (Fodor and Szabo 2005).

Excessive animal manure and fertilizer inputs do cause various environmental problems, including the accumulation and elevated leaching and runoff of nutrients (N and P) and heavy metal to ground water and surface water (de Vries *et al.*,2005). Groundwater contamination by nitrate-nitrogen and eutrophication of surface waters by phosphorus originating from land application of fertilizers and animal manure are well documented in some most populated areas like, Durres, suburb of Tirana, Lushnja etc.

Nonpoint source nutrient pollution of ground water and surface water by agriculture is a major, longstanding environmental issue in the United States (Sims 2005). As comprehensive nutrient, management planning has considerably widespread in the U.S., it has become increasingly apparent that the primary source of nonpoint nutrient pollution in many regions is nutrient imbalance. This is particularly true in areas where animal production has been geographically concentrated. Nutrient surpluses, usually due to imports of feed and fertilizer far in excess of exports in crops and animal products, lead to the buildup on nutrients in soils and increase the likelihood of poorly timed applications of manures. It is now widely accepted that a fundamental tenet of agro-environmental policy must be restoring nutrient balance on farms, especially those referred to as ‘concentrated animal feeding operations’ (CAFO-s). To achieve nutrient balance on farms or in watersheds requires a number of political, social, economic and logistic challenges.

Animal nutrition and Environmental problems

As a result of growing concern about the environment, intensification of animal production in many European countries is considered as potential source of air pollution and threat to soil and drinking water quality (Eeckhout and de Paepe 1994).

Nutritive ration of non-ruminant animals (pigs and poultry) contains 90% cereals seed (corn, wheat, barley, rye, and oat). The majority of “P” in cereal grains is organically bound as phytic acid or phytate. This form of P is nutritionally unavailable to non-ruminant animals due to the lack of phytase in their digestive tract. The P-excretion on the pigs and poultry faeces is potential source for soil and water pollution, due to his high level on the subsoil water and destruction of the ecosystem. In such situation the solution is utilization of phytase on the nutritive ration of pigs and poultry.

The current problems related to the phytase utilization are:

- The farmers have sometimes distributed the animal manure everywhere, as it is allowable by the law. In such situation the government has no control on the quantity of phosphorus and nitrogen in the arable soil and ground water. In Albania, the problems related to soil and water pollution are of great concern.
- On the other hand, some of the big companies, which produce feedstuff for animals, began four years ago to use the phytase in pigs and poultry nutritive rations. Most of the feedstuff is imported by neighbor countries like Italy, Greece, Republic of North Macedonia and Montenegro, and sometimes there is little known about the phytase.
- These companies produce meal and also pellet feedstuff for all animal categories. In consequence the possible destruction of phytase by pellet or heat treatment must also be considered.
- Because of the high price, most of the small farmers have no possibility to buy the feedstuffs from these companies, therefore to use the phytase on their farms. They are using Albanian cereals and legumes as feed for animals.

- Some of the pigs and poultry farms in Albania are in the suburb of the cities, but some others are near the living center, like as in Tirana, Durres, Vlore, Shkoder etc. The fecal excretion only by the poultry farms in Albania are nearly 41450 T per year.

Based on the data, Albanian livestock has increased from 31% to 50% of agricultural production since 1991. Dairy cows are raised throughout the country, while sheep and goats are common in the hills and mountainous areas while herds prevail in the south. While owning livestock is important for maintaining subsistence levels, profitability increases considerably with increase in herd size. On the other hand, it’s important to evidence that Albania is a mountainous country and only 16% of its territory is located at elevations of less than 100 m a.s.l. The agricultural land is distributed as follows: 43,3% in the plan or flat areas, 34% in the hilly zones and the remainder in the mountainous region.

The Table 1 reports the average quantity of organic fertilizer belongs to the species in Albania

Table 2 reports the composition of organic fertilizer in Kg/ton**.**

Table 3 reports about the N and P amended in the cultivated soils with manure.

Table 1. The average quantity of organic fertilizer belongs to the species in Albania (Piu and Locher 2001).

Species	**Live weight (kg)**	**The quantity (ton)/year**
	300	7
Cow	400	8
	500	10
Sow with piglets in maternity	200	2
Pigs	100	1.2
Piglets	30	0.9
Chicken	2	0.07
Sheep	40	0.6
Goat	30	0.4
Horse	500	6

Table. 2. The composition of organic fertilizer in Kg/ton **(**Piu and Locher 2001)

	N	P_2O_5	K_2O	Mg
Organic manure				
Cow manure	2	3	6	1.2
Horse manure	2.4	3	6.3	1
Sheep manure	3.2	3.3	8	0.8
Poultry manure	10	28	16	4
Pig manure	3.3	3.2	2.3	0.6
Liquid manure	6	5	5	3

Table 3. N and P amended in the cultivated soils with manure (Sulce and Veizaj 2006)

	Number of livestock	Annual manure production (in 000/tons)	N (tons)	P (tons)
Cattle	32 000	170 (35% moisture)	1300	380
Chickens	977 000	70 (60% moisture)	1200	300
Pigs	4 000	4 (45% moisture)	150	25
Sheeps/goats	40 000	20	600	100
Total			3250	805

Phytase supplementation as a way to reduce phosphorus excretion in non ruminant animals.

Interest in phytase for monogastric animals take place in regions, where soil and groundwater pollution due to animal wastes is a serious problem and phosphorus is a major concern. Indeed, plant phytate is the major form of plant phosphorus (Kirby and Nelson 1988) and phytate phosphorus itself has low availability.

The majority of the P in cereal grains and oilseed meals is organically bound as phytic acid or phytate. This form of P is nutritionally unavailable to non-ruminant animals due to the lack of phytase in their digestive tract. As result swine and poultry diets must be supplemented with highly available, inorganic sources of P to meet their P requirements. The poor bioavailability of P in the natural feedstuffs along with high dietary levels of supplemental P result in higher levels of fecal P compared with ruminant animals.

Studies in the early 1990s at Kentucky and Michigan showed that feeding pigs a low-P, corn-soy diet supplemented with phytase from a mutant strain of *Aspergillus niger* improved the bioavailability of P. At about the same time, research in the Netherlands also demonstrated the efficacy of phytase produced by recombinant *Aspergillus niger* in studies with pigs and chicks. In November, 1995 a commercial source of recombinant-produced phytase (Natuphos) was approved for use in the USA. Since then, other sources and forms of phytase have been developed and evaluated. From 1992 to 2001, 82 papers involving phytase were published in Poultry Science (N=55) and the Journal of Animal Science (N=27) with 48 of these published in the last 4 years. The studies clearly show that phytase increases the digestibility and bioavailability of P from phytate, reduces the amount of inorganic P needed to maximize growth and bone mineralization, and markedly reduces fecal excretion of P. Phytase seems to increase the bioavailability of Ca, Z, and other divalent cat ions that otherwise bind to phytate. Some studies suggest that phytase may improve ideal digestibility of amino acids slightly, but other studies have not shown this response. This new technology offers substantial benefits to swine and poultry production by reducing the potential for environmental problems associated with excess P excretion (Cromwell and Coffey 1991).

Information about the effect of microbial phytase use as a partial replacer of the bicalcium phosphate in the layers and weaned piglets, aiming a better utilization of the phytic phosphorus and decrease of the environment pollution could be found in (Piu *et al*., 2008). Utilization of microbial phytase (Natuphos) on the nutritive ration of weaned piglets (28 days old), was accompanied with improved performance parameters. In the experimental group, treated with Natuphos (750 FTU/kg), the excreted phosphorus amount in the dropping is decreased by 6,8%, while the excreted nitrogen amount is decreased by 5,2%. So, there is a positive output towards the minimizing of the environmental pollution with no degradable Phosphorus and Nitrogen.

Cromwell and Coffey (1991) concluded that a 50% reduction of excreted phosphorus would mean that 100.000 fewer tons of phosphorus would be excreted into the environment annually in the United States. Obviously, this could have a major impact and would be especially significant in countries, where livestock production is restricted because of environment pollution.

2. MATERIAL AND METHODS

Thirty-six piglets (Large White x Landras) of four litters were transferred to flat-decks and allocated to 2 groups (A and B) with 18 animals (9 males and 9 females), respectively. Two piglets from different litters (1 male and 1 female), with the same body weight were housed in every box (experimental unit). The litter origin was taken into account, avoiding that piglets from the same litter were allocated in the same treatment. There were nine replications per control group and nine also per treated group. The control group (A) was feed with a balanced diet, containing mono calcium phosphate. The experimental group (B) was feed with low level of phosphorus, without inorganic phosphorus. All the phosphorus in this group originates from soybean meal. This group was supplemented with NATUPHOS phytase 750 FTU/kg feed.

Ambient room temperature was maintained at 24^{0}C for three first weeks and lowered by 1^{0}C for each week thereafter. The ventilation also was provided to ensure good air quality. The basal diet mainly contained maize and soybean meal and the nutrient contents met or exceeded nutrient requirements recommended by NRC. The diets were offered ad-libitum and animals had free access to water.

Table 4. The calculated nutrient concentration of diet.

Nutrient concentration (g/kg feed)		
	Control group (A)	**Experimental group (B)**
ME (MJ/kg)	12.75	12.80
Crude protein	190.1	191.1
Crude fibre	38.4	38.1
Calcium	6.90	6.45
Phosphorus	6.0	4.2
Lysine	2.1	1.9
Metionine+Cystine	6.4	6.5

The six weeks of experimental period, Body Weight (BW), Daily Weight Gain (DWG) and Feed Conversion Ratio (FCR, kg feed/kg Body Weight Gain were measured weekly. Data are presented as arithmetic means with standard deviation of the mean (Mean ± SD). One-way analysis of variance and Student's *t*-test ($P<0.05$) were performed to test the differences between two groups.

3. RESULTS AND DISCUSSION

Feeding phytase NATUPHOS has slightly improved the production parameters respectively: Final Body Weight (FBW) by 5.6% and Daily Weight Gain (DWG) by 7.3%, compared with the control group.

Table 5. Efficacy of supplemented phytase in low phosphorus diet for piglets

Parameters		**Control group**	**Experimental group**
Production	n[1]	**X±SE**	**X±SE**
Initial BW, kg	16	12.2 ± 0.90	12.6 ± 0.48
Final BW[2], kg	16	23.2 ± 1.06	24.5 ± 0.96
DWG[3],g	16	369 ± 10.0	396 ± 7.33
FCR[4]	16	2.44 ± 0.11	2.43 ± 0.10

[1] Number of animals, (18 piglets/ every group, at the beginning of the experiment)

[2] BW at the end of the trial.
[3]DWG for whole experimental period. [4]FCR for whole experimental period.

Feed Conversion Ratio (FCR) was reduced (-4.1%) compared with the control group, but the differences were insignificant.

The supplementation of microbial phytase preparation (*Aspergillusniger*, NATUPHOS) 750 FTU/kg feed reduced the P-excretion by 20-25%, provided that pig's diets can be supplemented with an economical and efficacious level of phytase that will allow all of the supplemental inorganic P to be removed from the diet (Cromwell and Coffey 1991).

With the industrial production of phytase, application of this enzyme to pig's diet to increase P availability and improve animal performance, as well as reducing environmental pollution has gained widespread attention. The beneficial effects of supplementary phytases on P digestibility and animal performance have been well documented (Rao *et al*., 1999; Ravindran *et al*., 1999). The efficacy of any enzyme preparation depends not only on the type, inclusion rate and level of activity present, but also on the ability of the enzyme to maintain its activity in the different conditions encountered through the gastrointestinal tract and the conditions used for the pre-treatment of a feedstuff or diet.

4. CONCLUSIONS

Agricultural techniques and animal rest is a serious problem for soil and water pollution, especially in the areas with a big concentration of livestock farms. The situation is more problematic, when we add the fact that there is not government law to not allow the distribution of animal manure in everywhere. In such situation, it is difficult to have control on the quantity of phosphorus and nitrogen in the arable soil and ground water.

An original solution is the utilization of microbial phytase on the nutritive rations of non-ruminant animals. This could have a major impact in countries, where livestock production is restricted because of environment pollution. Nowadays, phytase supplementation is considered as a good way to reduce phosphorus excretion in non-ruminant animals.

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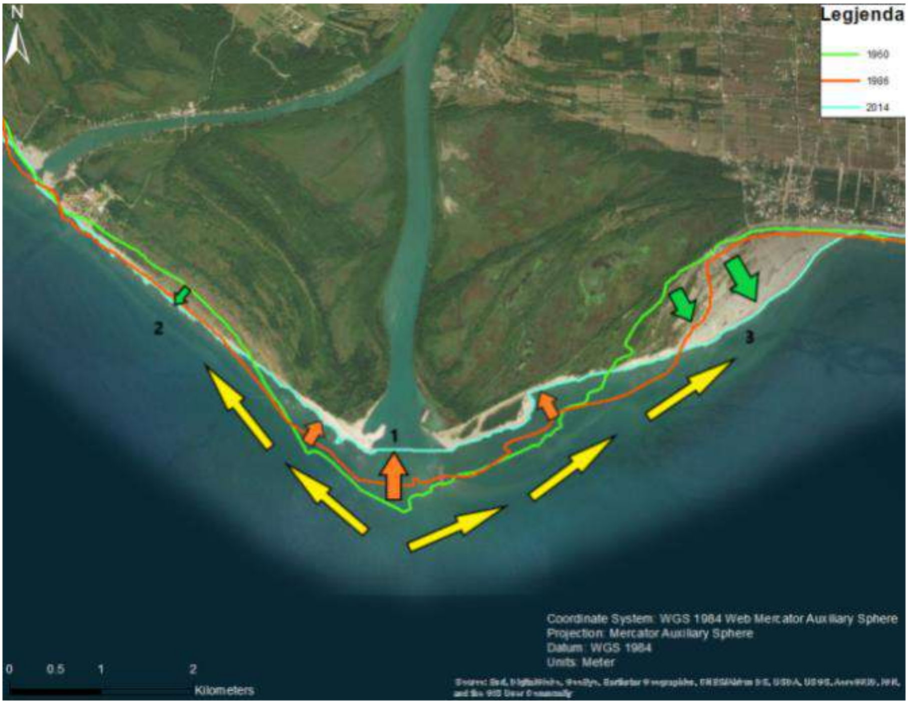
along Drini River in 1971 has disrupted this process. Reduced sedimentation and entrapment of sediment have exacerbated the intensity of coastal erosionin the Buna delta (Bego et al, 2012; Le Tissier, 2013; Faloutsos et. al. 2015), as well as Drini-Lezha delta (Boçi, 1994; Ciavola *et al*., 1995; Gjiknuri, 1995; Simeoni *et al*., 1997; Mathers *et al*., 1999; Fouache *et al*., 2001, 2010; Pano *et al*., 2003; Meçaj 2005). Consequentially, substantial coastline alterations are evident comparing imagery from the 1960-2014 timeperiod (Figure 2 and 3). In the last three decades, coastal erosion has led to the loss of “Franc Joseph” island, formerly situated at the very front of the Buna Delta (Dhora, 2017). *Part of the eroded material from the front of the delta gets redistributed along the shoreline, gradually leading to the flattening of the entire delta.*



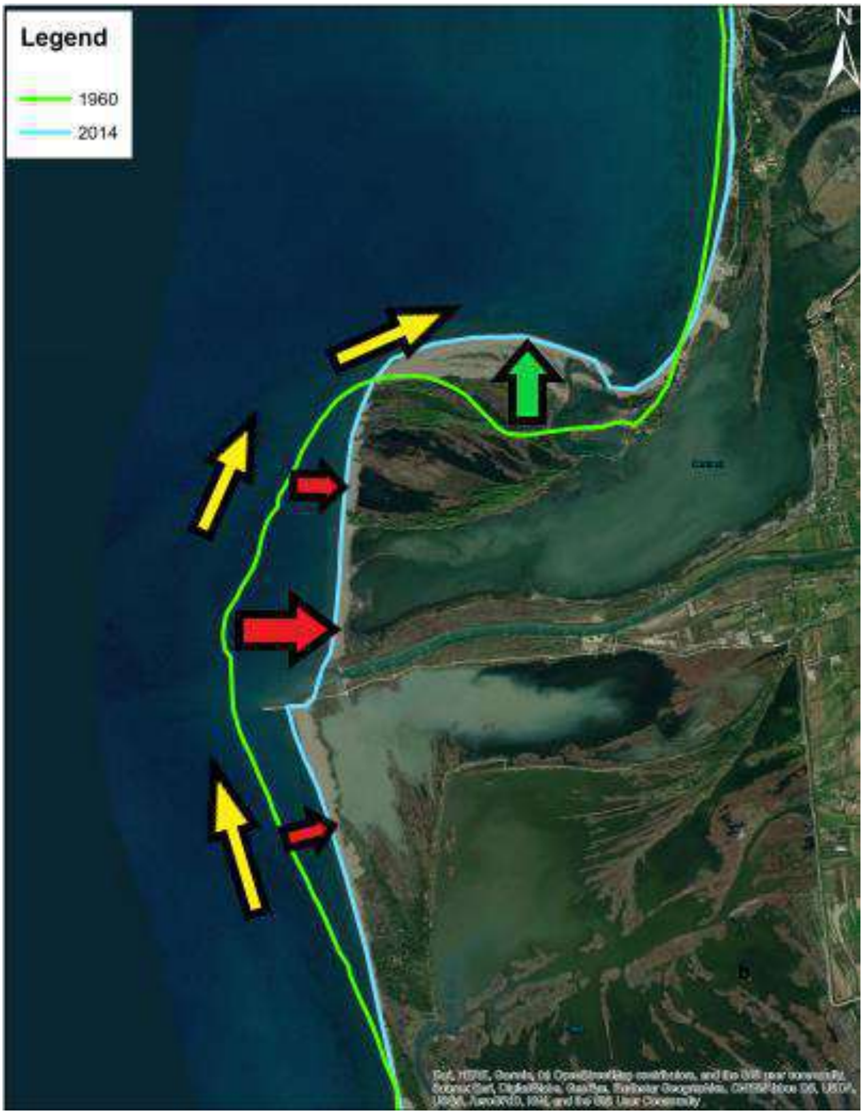


Fig.3: Coastline alteration in the Buna Delta (left) and Drin-Lezha Delta (right).

Coastal habitats have been subject to erosion and alteration since the construction of the first hydropower dams along the upstream of Drini River. Sand dunes are the most affected habitat type, having shrunken by about 63% across both Buna and Lezha deltas, because of the negative sedimentation budget. Coastal lagoons and estuars have also shrunken in size, with alluvial

HISTOLOGICAL ANATOMY AND EPIDERMAL CHARACTERISTICS OF LEAVES IN SPECIES WITHIN THE FAMILY *NYMPHAEACEAE*

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Abstract

This is the first study about the micromorphology of aquatic plants of the family Nymphaeaceae in Albania. These species have the status of rare and endangered and have medicinal and decorative values. Given the current status and importance, the present paper provides important information about the taxonomy and philogeny of Albanian flora. The two species here studied have been collected from the "National Park of Lura", Albania. The histo-morphological techniques, i.e. the colloidal technique and leaf epidermis detachment technique were employed. Leaf morphology and anatomical characteristics can serve as bioindicators of plant response to altered environmental conditions and changing habitat use. The present study reports about the micromorphology of leaf and indicators of epidermis of leaf in *Nymphaea alba* L. and *Nuphar lutea* (L.) Sibth. & Sm. Parameters of stomatal density, stomatal index, stomatal size, trichomes density, trichomes index and druses density were measured. As a result, leaf epidermis indicators such as stomata, trichomes and druses (crystals) show variations within the same species but grown in two lakes with different microclimates thus displaying intraspecific diversity in these species. The presence of staurocytic stomata, stomatal clusters and druses density in both species is for the first time reported.
Key words: Aquatic flora, bioindicator, pollution, leaf, micromorphology

1. INTRODUCTION

The family Nymphaeaceae includes perennial aquatic plants with floating leaves. There are two species of this family: *Nymphaea alba* and *Nuphar lutea* in Albania (Xhulaj *et.al.*, 2013). Leaf morphology and anatomical features can serve as bioindicators of plant response to altered environmental conditions and changing habitat use, particularly air (Pal *et. al.*, 2002;

Chaturvedi *et. al*., 2013) and soil (Abrams 1998; Tomasevic *et. al*., 2004; Sharma and Dietz, 2006). The morpho-anatomy of plant leaves can vary from contamination (Royer *et al*., 2008; Miller-Rushing *et al*., 2009; Pourkhabbaz *et al*., 2010). Differences in chemical composition and water depth, light intensity, and nutrient sediment conditions can lead to the genesis of specific morphotypes in plants that are not genetically determined and change rapidly with changing environmental conditions. Formal recognition of environmentally induced morphotypes has often resulted in an unwarranted evolutionary reversal and morphological sub-classifications of taxa (Kaplan 2002). In terms of *N. alba* species, its native population is declining rapidly in many European countries (Tomšovic 1988; Ejankowski and Małysz 2011). Despite the low number of European indigenous species, their high morphological polymorphism and plasticity have caused a persistent controversy regarding the divisions between taxa (Kabátová *et al*., 2014). As for *N. lutea* species, extreme morphological variability, uniform chromosome numbers and presumed hybridization have been obstacles to clearly understanding the relationships between taxa and to making a consistent taxonomic treatment (Padgett 2007). The epidermal parameters of the leaves, specifically the stoma and trichomes, their recognition and specification, serve the deeper characterization of a species and are an important indicator in determining the taxa in many plant families (Stuessy 2009). A change in stomatal density can be used as an indicator of environmental change (Case 2004). The presence or absence of trichomes and their density are influenced by various ecological factors, including tolerance to biotic and abiotic stresses (Bleeker *et. al*., 2012; Tian *et. al*., 2012). The specific mechanism that controls druses formation is unclear, but it has been suggested that a number of factors such as proteins, polysaccharides, and lipids or macromolecular membrane structures affect the shape and growth of crystals (druses). Druse may also have a purpose in regulating calcium (Horner and Wagner 1980; Arnott and Webb 1983; Webb, 1999). The different parameters of druse crystals are also related to the morphology of the leaves, as the density of these druse crystals is positively related to tissue density (Franceschi & Nakata, 2005). For these reasons, the micromorphological structure of the leaf and the stomata, trichomes and druses have been studied as an indicator of the relationship of *N. alba* and *N. lutea* species with the water ecosystem where they grow.

2. MATERIALS AND METHODS

Lura Lakes are a group of lakes of glacial origin, located in the eastern mountains of Lura and the Lura National Park in Albania. The aquatic plants studied here were collected in fresh condition, precisely from the “Liqeni i

Luleve” and the “Liqeni i Madh”. The coordinates of these lakes determined by GPS are: “Liqeni i Luleve” N 41044`25 "E 20011`52.9" with an altitude of 1572 m above sea level and “Liqeni i Madh” N 41 ° 47'24.3 "E 20 ° 11'51.2" with an altitude of 1716 m above sea level.

Optic microscope, Olympus microscope connected to C200 camera, camera, lugol, blumethylene, transparent nail polish, fresh leaves, scotch tape, razor are in the present study used.

The colloidal technique. Five species for each genus were collected per habitat, and the morphological features of the leaf were analyzed. The leaf epidermis was cleaned up with water and then letting it dry with water. Once dried up, in the middle of the sheet, on the upper and lower epidermis, it was placed transparent nail polish. Once the nail polish is dried, it is placed on top of the painted part of the scotch glue, then the glue that takes the traces of the stomata and trichomes from the sheet is removed, and placed on the lame. (Http://www.zoo.utoronto.ca/able/volumes/copyright.htm). All the leaves underwent this process. The microscope first with a small zoom, and then with a 40x zoom was used.

The detachment technique. It consists of detachment of the leaf epidermis with a scalpel or razor and placing the removed part on the blade, where a lugol or blumethylene dye has been previously applied. In our case for the study of stomatal types in the leaves of species *N. lutea* the dyeing technique has been modified for a clearer appearance and for this reason the stomata have been dyed first with lugol and then with blumethylene. Clean areas are photographed with a camera and a computer-connected camera (Johnston and Watson 1976; Hilu and Randall 1984). The preparations are placed under a microscope for observation.

The transverse cutting technique was employed for the anatomical study of the leaves.

The transverse technique. The leaves were transversely incised to study of tissues and the construction of canned organs (leaf). These incisions are made with a sharp razor and with the help of the spinal cord when they become perpendicular to the axis of the organ to be cut. The material (leaf) is placed between the two pieces of the marrow of the appendage, which helps to make thin cuts.

The dyeing technique. The cuts are transferred to a clock glass with distilled water, and then passed to a clock glass with safranin where they are left for 2-3 minutes to be colored. The same procedure is followed for dyeing with toluidine. After dyeing, the cuttings are sent back to a watch glass with clean water to be rinsed. After this action, the cuts are ready to be observed.

The preparation and observation. In the center of the blade we release a drop of glycerin, place the thinnest incisions and cover them with lamellae (Topuzi 2005). The Olympus microscope with a 40x lens was involved for the