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Study of haematology profile & histopathological changes in di-ammonium phosphate induced climbing perch, *Anabas testudineus* (Bloch.)

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Abstract
The current study includes the histopathological and haematopathological alterations induced by chronic (20 days) exposure of the fish Anabas testudineus to a sublethal concentrations (0.092 gm/L) of inorganic fertilizer, Di-ammonium phosphate (DAP). Behaviour response showed in test fishes like lost their natural colouration, loss of equilibrium, jerk movement before death. At haematological levels various parameters were analysed such as Hb, RBC, WBC decreases while in DLC, Neutrophil, Monocytes and Eosinophil values increase. For the histopathological study some vital organs were taken i.e. kidney, liver, intestine, testis and ovary. The present study showed major significant histopathological alteration like vacuolar degenerative changes, necrosis etc. found in liver, intestine, testis, ovary and kidney organs dysfunction in response to DAP toxicity effect in the fish A. testudineus. So, it is suggested that more suitable to culture at water fertilizer, Di-ammonium phosphate concentration of < 0.092 g/l for optimum growth performance. The information will be major role on different levels of responses of organisms with respect to pollutant stress is a necessary pre-requisite for the proper management of fertilizer application in agriculture and aquaculture.

Keywords: Di-ammonium phosphate, *Anabas testudineus*, histopathology, haematopathology, fertilizer, pollutant

Introduction
The Rainfall washes away fertilizers and other agricultural chemicals from widespread area. Natural waters are the ultimate recipients of fertilizer residues used for agricultural purposes which are transferred from land to water. Aquatic organism can survive in very low concentrations of the pollutants. But when these concentrations increased abnormally, they become fatal to the sensitive organisms like fishes (Awasthi *et al.*, 2008) [6].

Nitrogen pollution from agricultural sources is now considered to be a major problem in many regions of the world (Vidal *et al.*, 2000) [38]. The aquatic organisms are sensitive to environmental changes. Sub-lethal concentrations of fertilizers may cause ecological imbalance of these organisms after sufficiently long time of exposure probably as a result of cumulative impact of impaired metabolic functions (Abedi *et al.*, 2013) [1]. There is lacking in tissue level study of fertilizer pollution effects on aquatic fauna in such past review of literature.

Histopathological studies on aquatic fauna are a noteworthy and promising field to understand the structural organization that occurs in the organs due to pollutants in the environment. These structural changes vary with the body parts, nature of the pollutant, medium and duration of exposure. Water physio-chemical characteristics also influence histopathological manifestations of toxic effects (Galat *et al.*, 1985) [11].

Anabas testudineus (Bloch.), locally known as "kawai", which is an integral part of paddy field culture on this subcontinent, is also subjected to severe ammonia toxicity from ammonium fertilizers during the intensive fertilization of the crop fields.

Hence, in this paper efforts have been made to illustrate the haematological and histopathological alterations induced by this inorganic fertilizer, di-ammonium phosphate on the liver, kidney, intestine and gonads toxicity impact on air breathing teleost, *Anabas testudineus.*

Materials and Methods
The air-breathing teleost *Anabas testudineus* (Bloch.) procured and brought in container live from the local fish market, Darbhanga were washed with 0.1% $KMnO_4$ solution to remove dermal infection if any.

Healthy fish of average length (10–12 cm) and weight (30–34 g) were acclimated for 15 days to laboratory conditions. Commercial diet containing 28.58% crude protein was used through the experiment period with daily ration rate 3% of fish weight in the in morning (10.00 AM). Running tap water was used in all the experiments and the fish were adjusted to natural photoperiod and ambient temperature. No aeration was done and follows the methods of APHA (1985) [3].

Static acute bioassays were performed to determine LC_{50} values of Di-ammonium phosphate, the mortality was recorded after 24, 48, 72 and 96 hr, and were calculated by the Finney method (1978) [10]. The LC_{50} values for these periods were 1.10 g, 1.0 g, 0.97 g and 0.92 g respectively. 1/10th value of the LC_{50} value for 96 hr was taken as the sublethal concentration (Sprague, 1971). Twenty acclimated fish were exposed to a sub-lethal concentration (0.092 g) of Di-ammonium phosphate for 20 days. Side by side same number of fish as that of experimental one was maintained as the control groups. At the end of exposure period the fish were anaesthetized with 1:4000 MS 222 (tricane, methane, sulfonate, sandoz) for two minutes. At the end of exposure period the fish were anaesthetized with 1:4000 MS 222 (tricane, methane, sulfonate, sandoz) for two minutes. On 20th day blood samples were extracted from the caudal dorsal of the test fish. Estimation of haemoglobin, RBC, WBC, Lymphocytes, neutrophil, monocytes, basophil, eosinophil and determination of PCV (packed cell volume) as method (Akela *et al.* 1996; Shrivastav, 1979). Simultaneously, same day fish were taken out, sacrificed and the liver, intestine, kidney, testis and ovary were excised out and fixed in 10% Neutral Buffered Formalin for 18-24 hours fixed tissue samples were then processed and paraffin embedded tissue blocks were cut into serial sections (5-7 µ thick) by a rotary microtome and all the tissues was prepared using the standard histological methods (Luna, 1968) [15], stained with Haematoxylin and Eosin and microphotographs were taken.

Results

Behavioural response

The control fish shows a tendency to remain at the bottom of the aquarium with little disturbance. However, mortalities were removed immediately, and behavioural abnormalities were assessed at these regular intervals using a modified behavioural protocol checklist (Klesius *et al*. 2000) [13]. Scores were assigned daily to individual fish in the experiment and were based on the following scoring system: 0, no observed changes in behaviour; 1, swimming abnormally, lethargic or unresponsive, changes in skin coloration; 2, hyperactive or excitable, rapid operculation; 3, death. Mean behaviour scores were calculated per replicate treatment.

Just after introduction to test solution fishes showed increased swimming, surfacing and hyperactivity. Restlessness, rapid surfacing, peeling of skin and colour fading were prominent after 24 hr exposure. After 48 hr exposure the fishes showed slightly reduced activity and gradual increase in colour fading. Gill adhesion and a thin film of mucous were noticed on gills, operculum and general body surface at this stage. After 72 hr exposure increased surfacing and gulping of air was observed. At this stage fishes showed loss of balance and jerky movements during swimming.

The school formation, a characteristic of this fish, was found weakened in test animals as compared to controls at this stage. After 96 hr ulceration on trunk, base of caudal and pectoral fins were prominent in 95% of the animals. A thick film of mucous on whole body and gills was observed in almost all test fishes. Test fishes lost their natural colouration. Loss of equilibrium before death is a symptom shown all the test fish.

Haematological studies

From Table: –1 it is quite clear that Hb in control value is 11.56 ±0.06 gm/dl which is decreases under Di-ammonium phosphate as 6.79 ±0.11 gm/dl and showed highly significant ($P< 0.001$). Similarly the value of RBC decreases under Di-ammonium phosphate at 4.58 ± 0.08 in contrast to control value 6.39± 0.01. the decreased value under treatment showed highly significant ($P< 0.001$) (Table: –1, Figure:-1).values of Neutrophil, Monocytes, Eosinophil is increasing under treatment groups such as 16.1± 0.04, 8 ± 0.03 and 1.4± 0.02 in compare to control value such as 7.84± 2.01, 5 ± 0.05, 1.1 ± 0.03. Neutrophils are highly significant ($P< 0.001$), while Eosinophils showed significant ($P< 0.01$) and Basophil showed non significant ($P< 0.05$). In DLC (Differential leucocytes count) the values of Lymphocytes and Basophil are decreases under treatment groups. In control to values are 68.33± 242 and 1.1± 0.02 while under treatment the values decreases as 45 ± 0.02 and 1.3 ± 0.02. the Lymphocytes showed significant ($P< 0.01$) while Basophil showed non significant ($P< 0.05$). Similarly PCV (Packed Cell Volume) also decreases under treatment group as 12.01 ± 0.03 compared to control group as 35.97 ± 0.06. It showed significant ($P< 0.01$) (Table:- 1, Figure:-2).

At haematological levels various parameters, such as Hb, RBC, WBC decreases while in DLC, Neutrophil, Monocytes and Eosinophil values increases while Lymphocytes and PC value decreases. The increase or decrease value showed either significant, highly significant or non-significant. It causes various diseases Erythropoesis, anaemia, Leucocytopaemia, Neutropaemia, Lymphopaemia, Eosinophilia and Erythropaemia.

Table 1: Showing the effects of Di-ammonium phosphate on Hb, RBC, WBC, DLC, PCV of *Anabas testudineus*

Variable		Di-ammonium phosphate (96 hrs) exposure
Parameter	**Control**	**0.092 g/l**
Blood Hb (gm/l)	11.56 ±0.06	6.79±0.12 ***
TEC(RBC) (x 10^6µl)	6.39±0.01	4.58±0.08 ***
DLC (WBC) (% values)		
Neutrophil	7.84±2.04	16.1±0.04 ***
Lymphocytes	68.33±2.42	45.0±0.02 **
Monocytes	5.0±0.03	8.0±0.03 *
Eosinophil	2.0±0.03	3.0±0.02 **
Basophil	1.1 ±0.02	1.3 ±0.02 *
PC (%values)	35.97±0.06	12.01±0.03 **

Values are mean ± SE of 5 individual observations

* $P< 0.5$ Non Significant, ** $P< 0.01$ Significant, *** $P< 0.001$ Highly Significant

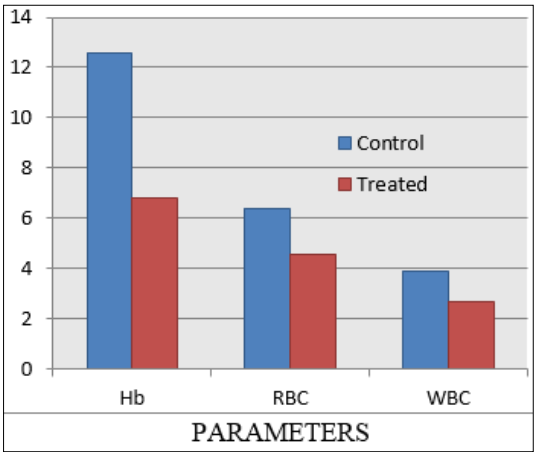

Fig 1: Showing the effect of Di-ammonium phosphate on Hb, RBC, WBC in *Anabas testudineus* (96 hrs) ****P*< 0.001

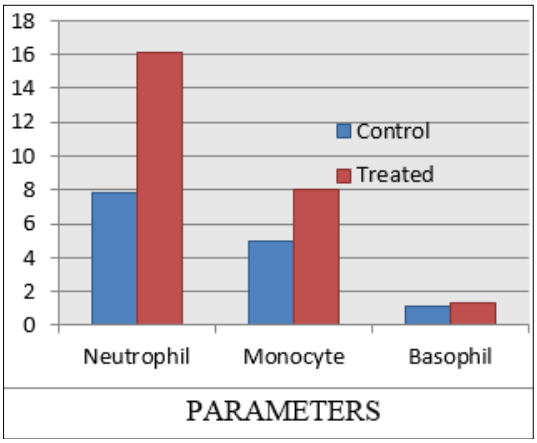

Fig 2: Showing the effect of Di-ammonium phosphate on Neutrophil, Monocytes, Basophil in *Anabas testudineus* (96 hrs) **P*< 0.05, *** *P*< 0.001

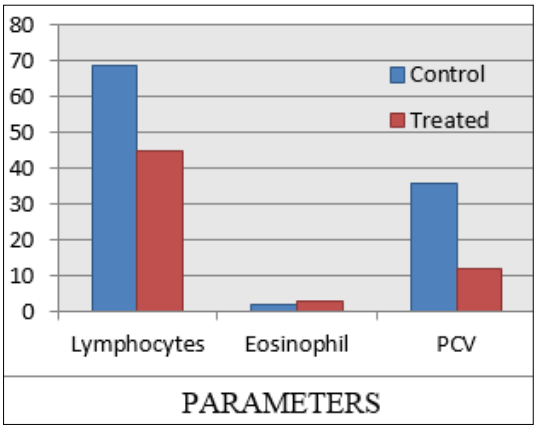

Fig 3: Showing the effect of Di-ammonium phosphate on Lymphocytes, Eosinophil, PCV, in *Anabas testudineus* (96 hrs) ** *P*< 0.01

Histopathology: Tissue samples liver, kidney, intestine, testes and ovary of *A. testudineus* were treated with sublethal di-ammonium phosphate concentration 0.092 g/l at 20 day after sacrificed and processed by conventional method, sectioned at 5-7 µm and stained with Haematoxylin and Eosin (Luna 1968) [15] and microphotographs were taken showed following major significant results:-

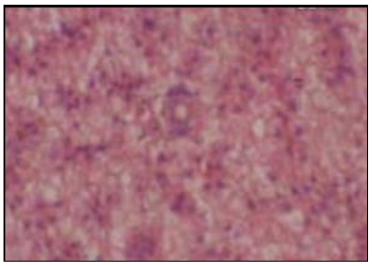

Fig 4.A: Photomicrograph of the normal liver of control fish, *Anabas testudineus*. H. & E., 100X

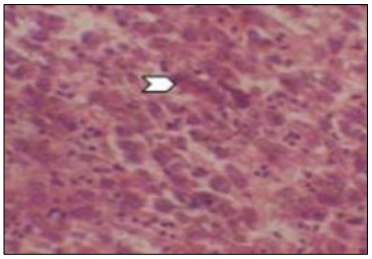

Fig 4.B: Photomicrograph of the liver of *Anabas testudineus* treated with DAP- 0.092 g/L for 20 days showing hemorrhagic liver tissue, blood congestion and necrotic cells. H. & E., 100X

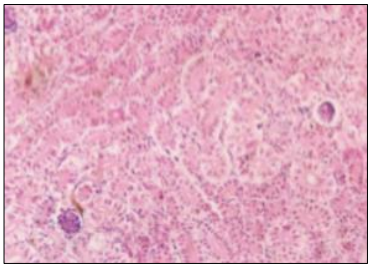

Fig 5.A: Photomicrograph of kidney of *Anabas testudineus* from control group showing normal. H.&E., 200X

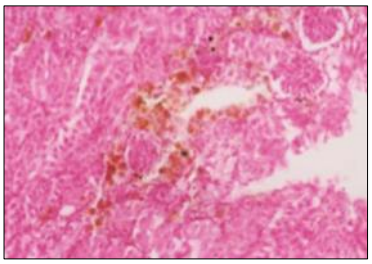

Fig 5.B: Photomicrograph of kidney of *A. testudineus* treated with DAP-0.092 g/l for 20 days showing degeneration of renal tubular epithelium, vacuolation and necrosis of renal tubules along with infiltration and necrosis of melanomacrophage center (arrow). H.&E., 20X

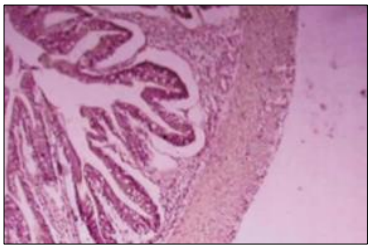

Fig 6.A: Photomicrograph of Intestine tissue of *A. testudineus* in control group showing normal appearance of circular muscles, longitudinal muscles, serosa and villi. H.&E., 120X.

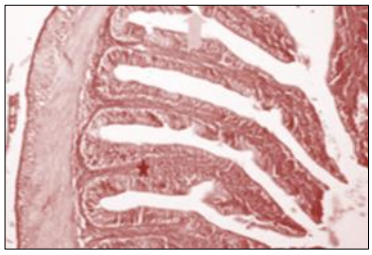

Fig 6.B: Photomicrograph of Intestine tissue of *A. testudineus* exposed to DAP- 0.092 g/L for 20 days showing desquamation (orange arrow) and mononuclear cell infiltration (MHI) (arrow). H.&E. 120X

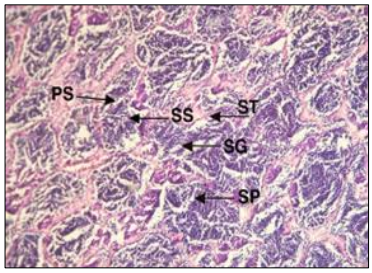

Fig 7.A: Photomicrograph of the testes of *Anabas testudineus* control fish showing sperm (SP), spermatogonia (SG), spermitide (ST), secondary spermatocyte (SS), primary spermocytes (PS). H.&E., 200X

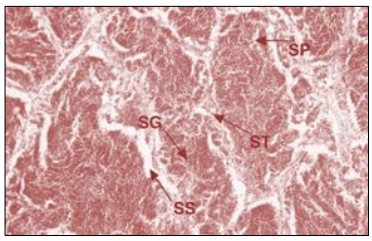

Fig 7.B: Photomicrograph of the testes of *Anabas testudineus* treated with DAP- 0.092 g/L for 20 days showing sperm (SP), spermatogonia condensation (SG), spermitide (ST), secondary spermatocyte vacuolation (SS). H.&E., 200x

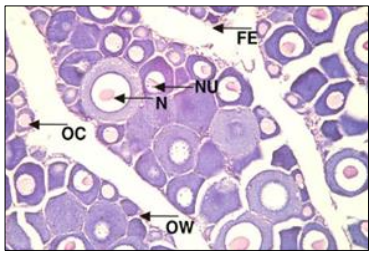

Fig 8.A: Photomicrograph of the ovary of *Anabas testudineus* control fish showing (OW) Ovarian wall, (FE) Follicular epithelium, (N) Nucleus, (NU) Nucleolus, (OC) Oocyte. H.&E., 200X

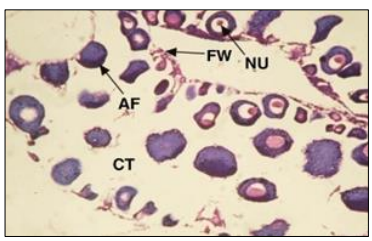

Fig 8.B: Photomicrograph of the ovary of *Anabas testudineus* treated with DAP- 0.092 g/L for 20 days showing (NU) Nucleolus condensed, (CT) Connective tissue degenerate (AF) Atretic follicle & (FW) Follicular wall disrupted. H.&E., 200X.

Liver: Histology of control fish groups were in normal structure. The liver is composed of hepatic lobule in which the central vein obscure. The parenchyma of the hepatic lobule is formed from hepatocytes which are arranged around the blood sinusoid in cord-like structure known as hepatic cell cord. There are bile ductile in between the cord of hepatic cells which are directed toward the periphery of the lobule to open in the bile duct (Figure:-4.A.). *Anabas testudineus* exposed to sub-lethal concentrations of DAP- 0.092 g/L for 20 days showed varied degree of hepatic cirrhosis as evidenced by vacuolization, space formation and resulting haemorrhage, vacuolar degeneration, necrosis, hyperemia and mononuclear cells filtration in portal regions were observed (Figure:-4.B.).

Kidney: The fish kidney consists of head and body kidneys. The head kidney is the anterior portion of the kidney and consists of lymphoid tissue. The epithelium becomes lower and more cuboidal in the intermediate segment. The distal convoluted tubules have epithelium with lightly eosinophilia and have no brush border (Figure:-5.A.), and kidneys displayed glomerulonephritis, vacuolar degenerative changes in the tubular epithelium and slight congestion (Figure:-5.B.).

Intestine: The intestinal wall of control fish, *A. testudineus* comprised of four distinct layers, *viz*. mucosa, submucosa, muscularis and serosa. The mucosal layer being thrown into finger like villi, which is made up of simple, long columnar cells and numerous goblet cells (mucous cells) with centrally placed nuclei. Sub-mucosa is thin and projected into mucosal folds constituting the lamina propria. This layer is composed of loose connective tissue with numerous collagen fibres and blood cells. Muscularis consists of inner, thick, circular, and outer, thin, longitudinal muscular layers. Serosa is formed of peritoneal layer and blood capillaries (Figure:-6.A.). In DAP exposed, marked histopathological changes in the intestine of *A. testudineus* have been observed in intestinal tissue, hydropic degeneration, necrosis and desquamation in epithelium cells at the apex of the villi were determined and mononuclear cell infiltration in the lamina propria was slightly observed (Figure:-6.B.).

Testes: Histology of normal testes shows the presence of healthy seminiferous tubules, which is internally lined by tubular epithelium which gives rise to spermatocytes. Testis of control fish were composed of lobules showing active spermat ogenesis. Sperm nests were found in majority of lobules (Figure:-7.A.). Fish exposed to sub-lethal concentrations of DAP- 0.092 g/L for 20 showed considerable degree of alteration in the histology of testes. In testes the seminiferous tubules are normally of varying shapes and sizes, each tubule has a definite thin fibrous wall which is not distinguished after spawning. It shows reduction in the number and condensation of spermatogonic cells as well as inflammation of cells, contraction and vacuolation of tubules (Figure:-7.B.).

Ovaries: Histology of control fish have thick ovarian wall with increased vascular supply and conspicuous blood capillaries. The connective tissue in the stromal was evident in good volume. The germ cells become associated with small epithelial cells more into cortex. The associated epithelial cells multiply and surround the germ cell which is now called oocyte developing into the stage I, stage II, stage III etc. and they will develop into the mature ovum which is nourished by the surrounding follicular cell (Figure:-8.A.). The present

investigation was undertaken to study the histopathological changes occurring in the ovary of *Anabas testiduneus* after exposure to sublethal dose of DAP- 0.092 g/L. Follicular cells are disrupted. Nucleolus shows condensation of crescent shaped dark granules at one side. Degeneration of epithelial cells causes vacuolation, breakdown of germinal vesical, many disrupted oogonia are the changes caused due to the exposure of ovary of *A. testudineus* to sublethal dose of DAP showing in (Figure:-8.B.).

Discussion

In the present study, certain deformities and unusual swimming patterns were found in fish exposed to 0.092 g/L and above concentrations. The results of the present study also indicate that the fish exposed to this fertilizer recover quickly when they were moved to freshwater. It is concluded that the fertilizers may have toxic potentials in the shallow water and therefore it should be carefully used in the areas closed to waterside. The responses recorded for the fish in this study are similar to those reported by other authors under various stress conditions (Paul and Banerjee, 1996; Rani *et al.*, 1997; Palanivelu *et al.*, 2005; Ufodike and Onusiriuka, 2008; Lata *et al*., 2008) [21, 24, 20, 35, 14]; Yanan, *et al.* (2015) [40]. Behavioural responses of fish to most toxicants are the most sensitive indicators of potential toxic effects (EIFAC, 1983) [9]. Acute toxic effect mercuric chloride was observed on zebrafish by Vutukuru, (2013) [39]; Pathak & Anand, (2020) [22]. The toxic effects of ammonium chloride fertilizer were reported on fish *C. batrachus*, by Sangeeta *et al.*, (2020) [26].

Haematological study

Haemoglobin: The present findings were in conformity of similar results of Hb decline has been also reported by Revathi *et al.* (2003) [25], Shipra *et al.* (2005) [27], Anwar and Choudhary (2009) [2], Arjun (2009) [4] has been reported in rat also. Again Pratibha and Kumar (2013) [23] has explained Hb treatment under mercury chloride and showed similar decrease level results in Hb and also showed Hb is highly significant ($P<$ 0.001).

RBC: The present study showed also conformity with *Heteropneustes fossilis*, the pesticide malathion resulted in a decrease in RBC count from 6,400,000 to 3,460,000/cm* in 96 hr at 7.6 ppm (Mishra and Srivastava, 1983) [17]. Recently Pratibha (2013) [23] has found similar decline nature of RBC under the treatment of mercury chloride to the fish *H. fossilis* (Bloch). The present findings, i.e. decrease in RBC level was close conformity with fish and mammals studies.

WBC: The present findings are conformity with Revathi *et al.* (2003) [25]; Shipra *et al.* (2005) [27]; Anwar and Choudhary (2009) [2]; Arjun, (2010) [5]. Pratibha, (2013) [23] has explained exposure of mercury chloride to the fish *H. fossilis*

DLC: During present study under DLC Neutrophil, Monocytes and Esnophil increase while Lymphocytes, Basophil decreases. The increase and decrease values are in close conformity with Muthalagi (2006) [18], Arjun (2010) [5] and Pratibha (2013) [23] under various exposure of sewage, chromium and mercury chloride to the fishes.

PVC: The present study is conformity with Muthalagi (2006) [18], Arjun (2010) [5] and Pratibha (2013) [23] in fish water fishes under exposure of sewage, chromium as well as cadmium chloride.

Histopathology

The liver of *A. testudineus* in the present study showed group exposed to the DAP showed hyperplastic hepatic and necrosis of hepatic cells. Similar observations were made in findings by (Tilak *et al.*, (2005) [33]; Ullah, *et al.* (2015) [36]; Mishra & Poddar (2016) [16]; Kalaiyarasi, (2017) [12]; Barbieri *et al.* (2019) [7] and Sangeeta, *et al.*, (2020) [26].

In our study, kidney tissues displayed glomerulonephritis and hyperemia after being exposed to different concentrations of sublethal ammonium chloride concentrations where the kidney is a one of the major organs of the toxic effects. Nayan (2012) [19] reported that degeneration of renal tubule epithelia, hyaline droplet degeneration, eventually may induce renal failure. Tilak, *et al.*, (2001) [34] also reported same in *Ctenopharyngodon idellus*. The above reporting is similar to the present observation.

The intestine is the most important organs in digestion and absorption of nutrients from food, and therefore, monitoring of these organs is considered necessary (Takashima, *et al.*,1982). Histological analysis of the digestive system is considered a good indicator of the nutritional status and toxicant ingestion of fish (Caballero *et al.*, 2003) [8]. All the pathological alterations showed a relationship with prevalence increasing with increasing di-ammonium phosphate concentration in present work.

Degeneration of epithelial cells causes vacuolation, breakdown of germinal vesical, many disrupted oogonia. Maximum damage is produced exposure of DAP in the ovaries of *Anabas testudineus*. Almost similar histological findings were reported by, Saxena and Garg, (1978). The histological abnormalities in ovaries may be caused by several factors *viz*. ionizing radiations, electric current, parasitic infections, xenobiotic toxicants and by a variety of effluents and aquatic pollutants (Shukla *et al*., 1984) [28], heavy metal on *Cyprinus carpio* by Vinodhini *et al.* (2009) [37]; fertilizer, ammonium chloride on fish *Clarias batrachus* by Sangeeta, *et al.*, (2020) [26]. The above reporting is similar to the present findings.

Testicular inflammation was documented as one of the common responses in both aquatic and terrestrial animals exposed to environmental toxicants (Sokal *et al.*,1985) [30], in term of vacuolization of tubular cells and distortion of somniferous cells along with inflammatory lesions. Shyni & Sreedhar (2014) [29] observed chronic effect of urea on testicular structure of the black clam. Similar effects observed in application of ammonium chloride on fish *Clarias batrachus* by Sangeeta, *et al.*, (2020) [26]. The above reporting is similar to the present findings.

Conclusion

It could be concluded that *Anabas testudineus* with average weight 30.0± 4.0 g, exposed with fertilizer, Di-ammonium phosphate, 0.092gm/l, at histopathological observation were found the liver showed vacuolar degeneration, necrosis, hyperemia, kidneys displayed glomerulonephritis, vacuolar degenerative changes in the tubular epithelium and slight congestion, intestine showed necrosis and desquamation in epithelium cells at the apex of the villi, sperm showed very significant histopathological changes, condensation of spermatogonic cells as well as inflammation of cells, contraction and vacuolation of tubules and while ovary showed degeneration of epithelial cells causes vacuolation, breakdown of germinal vesical. At haematological levels various

parameters, such as Hb, RBC, WBC decreases while in DLC, Neutrophil, Monocytes and Eosinophil values increase.

So, it is suggested that more suitable to culture at water fertilizer, Di-ammonium phosphate concentration of < 0.092 g/l for optimum growth performance. The information will be major role on different levels of responses of organisms with respect to pollutant stress is a necessary pre-requisite for the proper management of fertilizer application in agriculture and aquaculture.

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