

Chapter 2

Methodology

Aquatic toxicology is the scenario of study to identify the risk to the target and non-target organs of any aquatic organism arising from the continuous exposure to the stressor or industrial effluents, pesticides, heavy metals, municipal wastes, etc. Toxicity testing on higher animals is neither possible nor profitable; in view of this, toxicity tests are being carried out on small animals like freshwater fishes, which are more sensitive to the water quality. Environmental pollution including air, water, and land is due to accumulation of heavy metals, effluents, or pesticides in one or other forms, generated during manufacturing or application processes in industries.

The comfort of life enjoyed by our society can often lead us to forget how lives can be dramatically changed by unseen hazards. Chemical and biological contaminants of water supplies can be pivotal by causing diseases and even be fatal to life in water and on land.

Keeping in view the severity of an aquatic toxicity, in the present investigation, fish *P. reticulata* was exposed to the tannery industrial effluent for 96 h. Only acute toxicity experiments are carried out for the toxicity studies.

Acute toxicity tests have played an important role in man's effort to monitor and study the effect on the biota. The terms toxicity test and bioassay are frequently interchangeable.

Test fishes were collected from Kham River, 2.5 km away from Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, and from the water pools at Khultabad, 20 km away from Aurangabad, on the way to the famous historical destination, *the Ellora Caves*.

Tannery effluent samples were collected from the tanning industries situated at Paithan, 50 km away from Aurangabad. Effluent was collected and brought into the laboratory, and grades of dilutions were prepared as 10%, 20%, 30%, 40%, and 50%.

Fishes were brought to the laboratory, and special care was taken to avoid any mechanical injury while carrying to the laboratory. Fishes measuring 2–7 cm and weighing 2–5 g were selected and were acclimatized for 15 days under the laboratory conditions before the experimentation.

Before the exposure and during the experiment, feeding was completely stopped. Five aquaria of 10 L capacity were chosen for the experiment to avoid the overcrowding of fishes. In each aquarium a batch of ten fishes were exposed in the series of ascending dilution of the effluent from 10% to 50%.

Water in the aquaria was changed every day after 24 h. In the meantime, the set of control fishes was maintained simultaneously, in a separate aquarium with effluent free freshwater.

Mortality in the experimental fishes was keenly observed, and TLM is recorded at 50% death in fishes.

The fishes, which survived, were dissected to obtain the pituitary gland and gonads for the cellular observations.

After dissection the tissues like gonads and pituitary gland were fixed in Bouin's fluid for 24 h.

2.1 Histological Procedure

2.1.1 Fixation

It is important that the original structure of the tissue is preserved before it reaches the histopathology laboratory. As they die, cells break down releasing enzymes from their lysosomes and other intracellular organelles, which start to hydrolyze components of the tissue—a process termed autolysis. The degree of breakdown depends on the tissue and what has happened. For example, postmortem tissue is not usually taken until several hours or days after the person has died, and this would have undergone much more autolysis than a surgical specimen. Different components of tissue vary in their sensitivity to enzymatic digestion—RNA is particularly sensitive, most proteins less so, and DNA may remain intact for very long periods. Cells and soft tissues tend to be more susceptible to breakdown than bone, cartilage, tendons, and proteins of the extracellular matrix. A histologist has to be aware of all these processes and distinguish between changes that are due to a disease and those due to tissue autolysis.

Hydrolysis is the breakdown of proteins and nucleic acids, by reaction with water. These reactions are generally slow, but occur quickly if catalyzed by enzymes. Proteins are hydrolyzed by proteases; DNA and RNA are hydrolyzed by nucleases.

To minimize tissue breakdown, samples are often placed in a solution of fixative. However, since different fixation procedures are appropriate for each staining technique, it is important to know what technique will be applied to a tissue when it is taken. In addition to preventing autolysis, fixation may serve to retain the structure of the tissue and limit microbial growth. The most widely used fixative for light microscopy is 4% formaldehyde (formalin).

Another approach is the use of reagents such as ethanol, methanol, or acetone, which disrupt hydrophobic bonds and protein structure and remove water from the

tissue. There are critical differences between these two classes of fixative; aldehydes destroy amine groups, but tend to maintain tissue structure well: alcohols usually result in poorer preservation of structure (because they dehydrate cells) but do not destroy amine groups, and they can preserve some secondary structure in proteins (OpenLearn. The open University 2016).

2.1.1.1 Fixative

Bouin's Fluid

It is a water-based fixative and can be used for different soft tissues of animals.

Preparation of Bouin's Fluid Fixative

Take roughly 20 g of picric acid in 1000 mL of water, stir properly, and filter the solution:

Aqueous picric acid, 75 mL.

Formaldehyde 40%, 25 mL.

Glacial acetic acid, 0.5 mL.

Tissues were fixed for 24 h in Bouin's fluid.

2.1.2 Washing

After fixation the tissue turns completely yellow, a result of the picric acid. The yellow color of picric acid will disturb the staining of the slides affecting its color resolution, and for that reason it has to be removed.

If the color of picric acid is removed, then the stains will take effect. To remove picric acid color, the tissue has to be washed in 70% alcohol. If the color does not disappear within few minutes, you can keep the tissue in 70% alcohol for hours; this will not damage the tissues.

After placing in 70% alcohol, the tissues were transferred to lithium carbonate solution to remove the remaining picric acid from the tissue. Here also we can keep the tissues overnight. We kept the tissue overnight in lithium carbonate solution, and the yellow color of picric acid has completely disappeared, leaving the tissue fresh white in color.

If the tissues are to be embedded in wax, then water must be removed from the cells of the tissue, which is done by dehydration with alcohol grades. The tissues were passed into a series of alcohol grades for dehydration:

30% alcohol, 15 min.

50% alcohol, 15 min.

70% alcohol, 15 min.

90% alcohol, 15 min.

100% alcohol, 15 min.

Xylene, 5 min.

The gonads and pituitary gland of guppy are very small, so the tissues were kept for 15 min in alcohol grades; the time can be increased if the tissues are of larger size. The tissues are kept in xylene in the last step as after washing cold impregnation is to be followed in which xylene is used.

The tissue has to be embedded in solid medium; once the tissue is impregnated in a solid medium, it can be used later when we like.

The tissue was then processed for cold impregnation.

2.1.3 Cold Impregnation

Solid paraffin wax was cut down into small pieces in a 100 mL beaker, and 50 mL xylene was added to it. The mixture was left for 2 h untouched. After 2 h stir this solution thoroughly so that the mixture converts into a paste.

The tissues were then transferred to the wax xylene paste for 12 h.

2.1.4 Hot Impregnation

The tissue is embedded in a solid medium by the help of first removing the tissue water which is then replaced by any solid medium such as paraffin wax so that the tissue is rendered firm enough to enable thin sections to be cut; at the same time, the tissue is soft (not so hard) to enable microtome knife to cut the sections. The embedding medium has to thoroughly permeate the tissue in fluid form so that it solidifies without any damage to the tissue. The most satisfactory embedding medium used in routine histology is paraffin wax (<http://www.rajswasthya.nic.in/RHSDP%20Training%20Modules/Lab.%20Tech/Histo/Introduction.pdf>).

Solid paraffin is taken in a beaker of 500 mL and put in the oven (Fig. 2.3) for melting at 58 °C. After complete melting of the paraffin wax, it was filtered, and tissues were transferred into it and kept for 1.5–2 h.

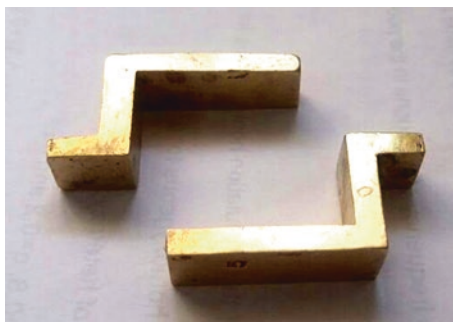
2.1.5 Block Preparation

Two “L” blocks (Fig. 2.4) of stainless steel were taken with glycerin applied in its inner side so that the wax does not stick with it and the block can be freely removed.

Fig. 2.3 Oven used for paraffin wax melting and hot impregnation



Fig. 2.4 L blocks



L blocks were placed on a wooden table, and molten paraffin wax was poured in it. After few seconds the tissues were placed in these L blocks and kept untouched for 2–3 h.

After the complete solidification of wax, the L blocks were removed, and a square wax block with the tissue in it was ready for trimming.

2.1.6 Trimming

To get exact square blocks of desired size, the block was trimmed with a cutter in a perfect square which was fixed on the holder of the microtome with the help of molten wax.

Fig. 2.5 Microtome



2.1.7 Microtomy

Blocks were cut on microtome (Fig. 2.5) at 7 μ thickness. Generally the block cutting was done when temperature is moderate or low; at high temperature the wax ribbon does not give fine sections.

2.1.8 Mounting the Ribbon on Slides

The ribbons formed after cutting the wax blocks with tissues are placed on a large filter paper. The slides were washed and cleaned to remove the grease. The mounting of the tissue was done; the tissue must stick firmly on the slide, and to achieve this, the slide was layered with egg white. Egg white was taken in a small beaker of 50 mL, and 10 mL water was added to the egg white. Water was added in the egg white as the hematoxylin stain would also stain the white egg layer.

The ribbon and sections have to be spread on the slide evenly without any fold in the sections. To achieve this the ribbon was placed on the slide, and few drops of water were placed between the sections and the slides with the help of a dropper. Then the slide was placed above the spirit lamp for 1 s; this was repeated for three to four times till the sections were spread uniformly on the slide.

The slides were kept to settle and dry for 2 h which was followed by the staining of slides.

2.1.9 Staining Procedures

Stains used for staining the pituitary gland.

2.1.9.1 Tinctorial Methods

Many stains have been developed over the years which demonstrate cell types in the neuroendocrine complex. These stains are empirical dye and have been used particularly to demonstrate pituitary and pancreatic cells. It also gives indication of the cells responsible for the synthesis of specific hormones related to maturation, ovulation, parturition, etc.

2.1.9.2 Preparation of Stains for Pituitary Gland

Constituents of MTS (*Mallory's triple stain*).

First component of MTS:

Acid fuchsin, 1 g.

Distilled water, 100 mL.

Mordant

Phosphotungstic acid, 1 g + distilled water, 100 mL.

Second component of MTS:

Distilled water, 100 mL.

Aniline blue, 0.5 g.

Orange G, 2 g.

Oxalic acid, 2 g.

2.1.9.3 Preparation of Stains for Gonads

Harris Alum Hematoxylin:

Hematoxylin, 1 g.

Absolute alcohol, 10 mL.

Ammonium or potassium alum, 20 g.

Distilled water, 200 mL.

Mercuric oxide, 0.5 g.

Glacial acetic acid, 8 mL.

Dissolve the hematoxylin in absolute alcohol and the alum previously dissolved in hot distilled water. Heat the mixture to boiling point and add the mercuric oxide. Cool rapidly and filter the stain. After cooling add acetic acid. The stain is ready for use. (Generally the stain is kept for 1–6 months for maturation, but nowadays ready-made stains are used.)

Eosin

1% Stock Alcoholic Solution:

Eosin, 1 g.
Distilled water, 20 mL.
Dissolve and add alcohol 95%, 80 mL.

Working Solution:

Take stock solution (1 part) and add 80% alcohol (3 parts). Just before use add 0.5 mL glacial acetic acid to each 100 mL of stain.

Once the stains are prepared, then following the staining chart, the slides were stained:

Xylene, 3 min.
100% alcohol, 5 min.
90% alcohol, 5 min.
70% alcohol, 5 min.
30% alcohol, 5 min.
Hematoxylin, 20 min.

One Water Dip:

Eosin, 15 min.
30% alcohol, 5 min.
50% alcohol, 5 min.
70% alcohol, 5 min.
90% alcohol, 5 min.
100% alcohol, 5 min.

Xylene

Lastly mounting with the help of DPX was done using cover slip.

References

OpenLearn. The open University (2016). <http://www.open.edu/openlearn/science-maths-technology/science/biology/introduction-histology/content-section-2.1>

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