TOXIC SUBSTANCES & THEIR EFFECTS ON FISHES

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Let us first understand what the Toxic substances are? And how they differ from substances having almost similar meaning?

- "Toxicants" are the substances that are produced by or are a by-product of anthropogenic activities.
- A "contaminant" is any physical, chemical, biological or radiological substance or matter in water (The Safe Drinking Water Act (SDWA)).
- "Xenobiotics" are "foreign substances", that are, displaced from their s normal habitat and foreign to the organism.
- A "pollutant" is a substance or energy introduced into the environment that imparts undesired effects, or adversely affects the usefulness of a resource.
  (US EPA(United States Environmental Protection Agency).

Toxicity

Toxicity involves the reversible or irreversible disruption of normal biochemical processes, which may result in impairment or loss of cell viability and regenerative capacity. In extreme cases, whole organs may fail and the organism may die.

Localized acute toxicity usually affects organs responsible for absorption and elimination due to such factors as the presence of particular enzymes, local blood supply, and the organ's regenerative capacity. These include the skin, stomach, liver, intestines, lungs and kidneys. This kind of toxicity is named after the organ or tissue affected, e.g., nephrotoxicity for the kidneys and hepatotoxicity for the liver.

Systemic toxicity may take the form of carcinogenicity, impaired immunity, changes in body weight, etc**. (**Tisserand, R., & Young, R. (2014)**.**

Toxicity testing

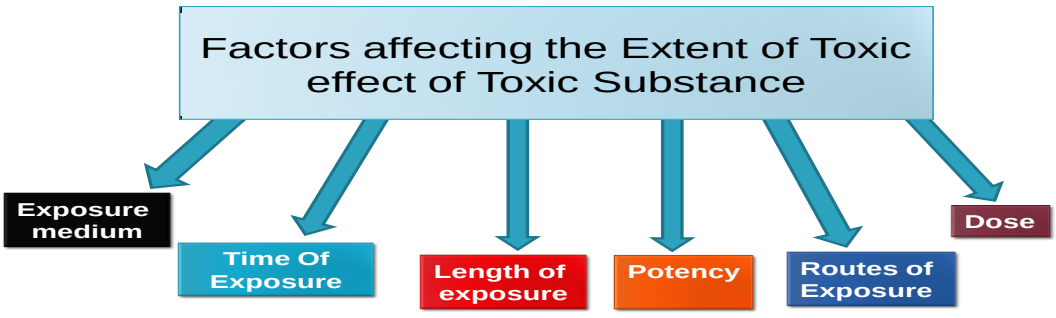
- A dose threshold is a dose level below which no observable effect occurs
- **LD_{50} (effective dose)** is the dose causing 50% lethality in an animal population. The LD_{50} is often given as a measure of acute toxicity of chemicals. The higher the LD_{50}, the lower is the acute toxicity. A highly toxic chemical (with a low LD_{50}) is said to be potent.
- A **LC_{50} (Lethal Concentration)** is the lethal concentration required to kill 50% of the population.

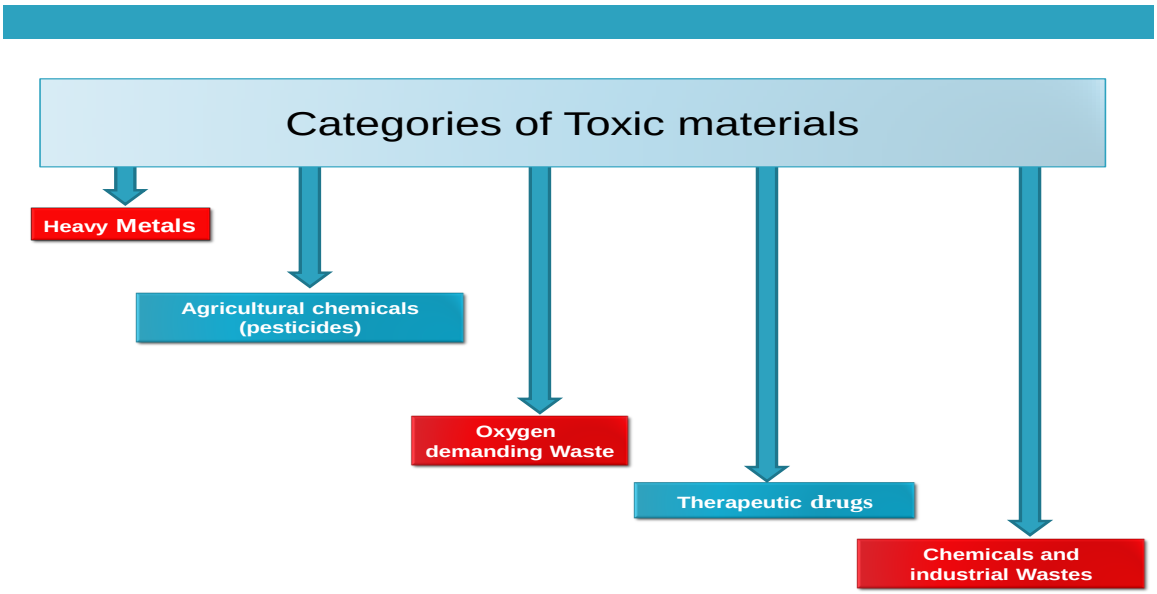
- The **EC_{50} (Effective Concentration)** is the concentration of a drug that gives half-maximal response.
- **ED_{50} (effective dose)** is the dose causing a specific effect other than lethality in 50% of the animals.
- The **IC_{50} (Inhibitory Concentration)** is the concentration of an inhibitor where the response (or binding) is reduced by half.

Factors influencing toxicity:

1. **Composition of the toxic agent**
2. **Dose and Concentration:** (a) Exposure medium, (b) Length of exposure, (c) Time of exposure and (d) Route of exposure
3. **Metabolism of toxicant,** 4. **Gender,** 5.**Age**

- Toxicological tests measure the effects of a limited exposure of an animal to a substance (acute toxicity) as well as repeated, long-term exposure (chronic toxicity).
- Substances are also tested for more **specific endpoints** such as **cytotoxicity** (ability to damage cells), **mutagenicity** (ability to cause changes in genetic material), **carcinogenicity** (ability to cause cancer), and **teratogenicity** (ability to cause birth defects).





Major Toxicants and effects of their exposure in fishes - Heavy Metals & Pesticides :

1. Heavy metals:

- Heavy metals are defined as metallic elements that have a relatively high density compared to water e.g., arsenic, lead, mercury, cadmium, chromium and some 'trace elements' like copper, selenium and zinc.
- They play an important role in maintaining the body metabolism. But if present in quantity more than required by the body, heavy metals can prove to be damaging.
- Urbanization and industrialization have increased dumping of heavy metals into the environment
- As fish is in direct contact with water, they are therefore used as biomonitors to assess the bioaccumulation and biomagnification of contaminants within the water system and tell us the health of other aquatic animals.

Table 1. Maximum Permissible Limit (MPL) in ppm (parts per million) of Heavy Metals in Fish.

Heavy Metals	Maximum permissible limit	References
Cadmium	0.05	FAO, 1983
Zinc	30	FAO, 1983
Copper	30	FAO, 1983
Chromium	1	FAO, 1983
Iron	100	WHO, 1989
Mercury	0.5	WHO, 1989
Nickel	0.05-5.5	FAO, 1983
Lead	0.5	FAO, 1983
Arsenic	1.4	WHO, 1989

Table 2. Ill effects of major heavy metals:

Heavy metal	Effects on fish	Effects on humans	References
Iron (Fe)	Disruption in respiration; reduction in the area of gill accessible for respiration, damaging the respiratory epithelium and death due to suffocation		Dalzell et al., 1999;
Mercury (Hg) and Methylmercury (MeHg)	Liver, gill arches, blood parameters, kidney, nervous system and olfactory epithelium damage, reduces sperm viability, production of eggs and their survival	Gingivitis, congenital malformations and spontaneous abortion; damage to CNS and brain; abortion.	Baatrup,1991; Oliveira Ribeiro et al., 1985; Oliveira Ribeiro et al., 2006; Raldúa et al., 2007; Pandey et al., 2014

	rate		
Nickel (Ni)	**Cause respiratory problems**		**Palaniappan et al., 2004**
Arsenic (As)	**Death because of suffocation; suppression of antibody levels; changes in functioning of T cells and B cells and making them inclined to infections**	**Stomach, lung and intestine irritation, decreased production of erythrocytes and white blood cells and skin disturbances; cause infertility; death**	**Ghosh et al., 2006; Liao et al., 2014; Pandey et al., 2014**
Chromium (Cr)	**Hinder the behavior and growth of fish; spoiling**	**Can irritate skin and can produce ulcer; cause liver,**	**Vinodhini et al., 2008; Benoit, 1976; Pandey et al.,**

	of the respiratory epithelium of gills and death	**kidney, circulatory and nerve tissue damage**	**2014 Awasthi et al.,2019 Awasthi et al., 2018**
Copper (Cu)	**Vacuolization of endothelial cells and hepatocytes; shrinkage and necrosis in liver; decreased egg production; spawning blockage; decreased survival of young; low fertility**	**At higher doses, it causes damage to liver and kidney, anemia, and intestinal and stomach irritation; Wilson's disease**	**Arellano et al., 1999; Figueiredo-Fernandes et al., 2007; Sorensen, 1991; Pandey et al., 2014**
Cadmium (Cd)	**Myocardial diseases have been observed**	**Lung cancer, obstructive pulmonary disease, osteomalacia and osteoporosis**	**Pandey et al., 2014**

Zinc (Zn)	**Affects tissue respiration in some fishes; hypoxia; accrues structural damage affecting the growth, development and survival of fish; affects hatchability**	**Relatively harmless; long-term and high-dose interferes with the copper uptake**	**Kori-Siakpere et al., 2008**
Lead (Pb)	**Genotoxic; cytotoxic damage in gill and fin epithelial cells in some fishes; in other fishes, it delays embryonic development, inhibits growth, suppresses reproduction, causes kidney dysfunction;**	**Effects depend on dose and age; high level causes problems in the synthesis of hemoglobin (Hb) and causes chronic damage to nervous system**	**Ibemenuga, 2013; Pandey et al., 2014**

	inhibits hatching of eggs, hypertrophy of gills		

Heavy metal accumulation in fishes:

Kumar et al., 2019 studied accumulation of heavy metal in fishes of different strata of the Ganges at Kanpur, U.P. India and concluded that accumulation of these metals potentially leads to toxicological manifestations among fishes, as evidenced by following figure (Fig. 4).

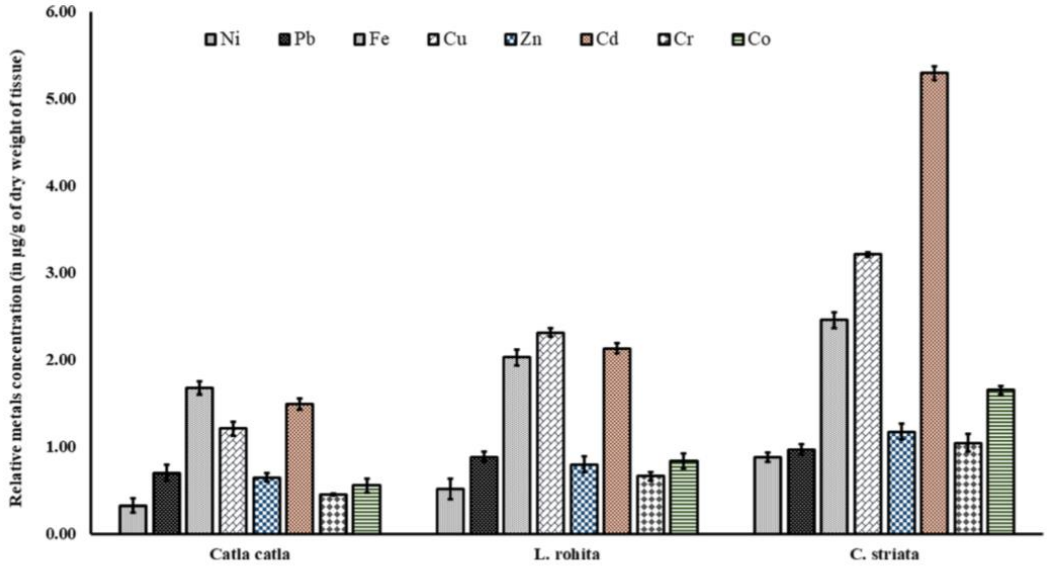


Fig. 4 Concentration of metals (µg/g) in fish liver tissue

2. Pesticides

Any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any insects, rodents, nematodes, fungi, or weeds, or any other forms of life declared to be pests; any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant." --**Federal Insecticide, Fungicide, and Rodenticide Act (US EPA)**

Pesticides can be grouped according to the types of pests which they kill:

- Insecticides - insects
- Herbicides - plants

- Rodenticides - rodents (rats and mice)
- Bactericides - bacteria
- Fungicides – fungi
- Larvicides - larvae

The US EPA has developed “category use” definitions of pesticides based on extent of their toxicity.

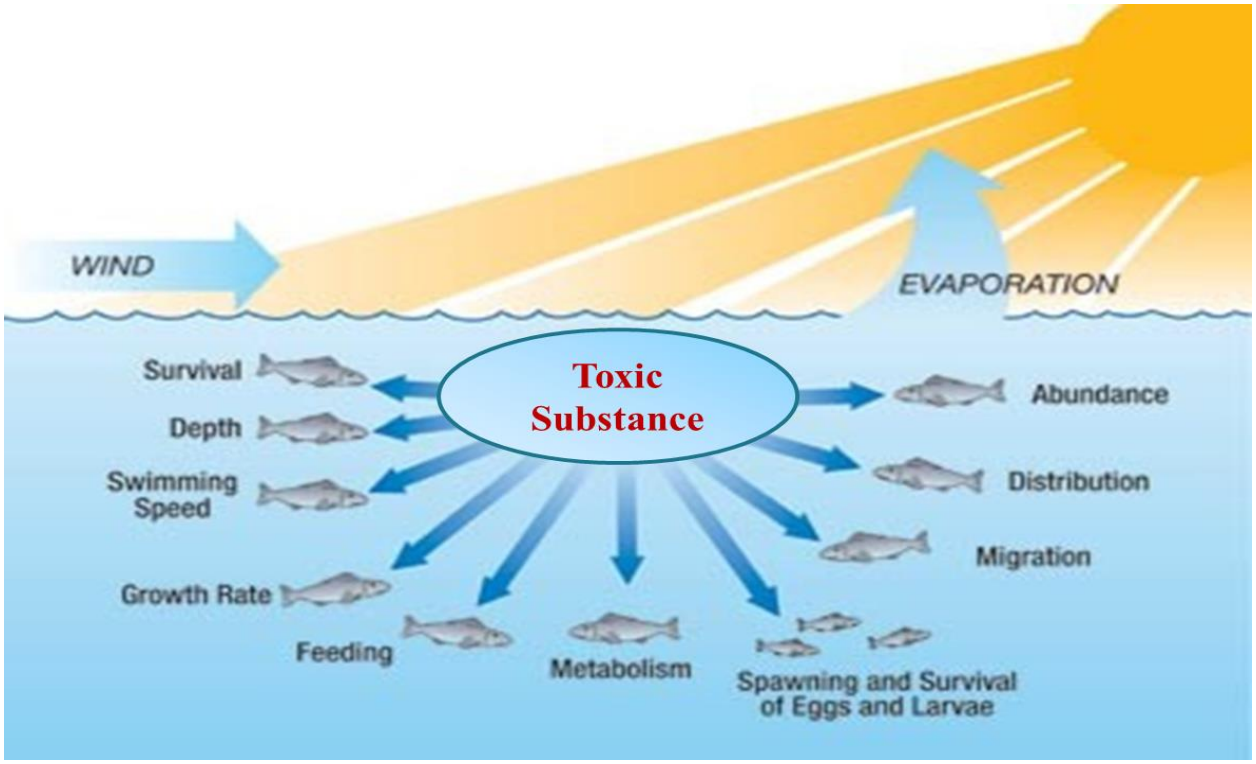
- **Category I pesticides** are highly hazardous, are classified as restricted use and have an oral LD50 less than or equal to 1.0/kg of body weight;
- **Category II pesticides** are moderately toxic and have an oral LD50 less than or equal to 500 mg/kg;
- **Category III pesticides** are generally nontoxic and have an oral LD50 less than or equal to 15,000 mg/kg.

In addition the US EPA has also developed a “carcinogenicity categorization” to classify pesticides.

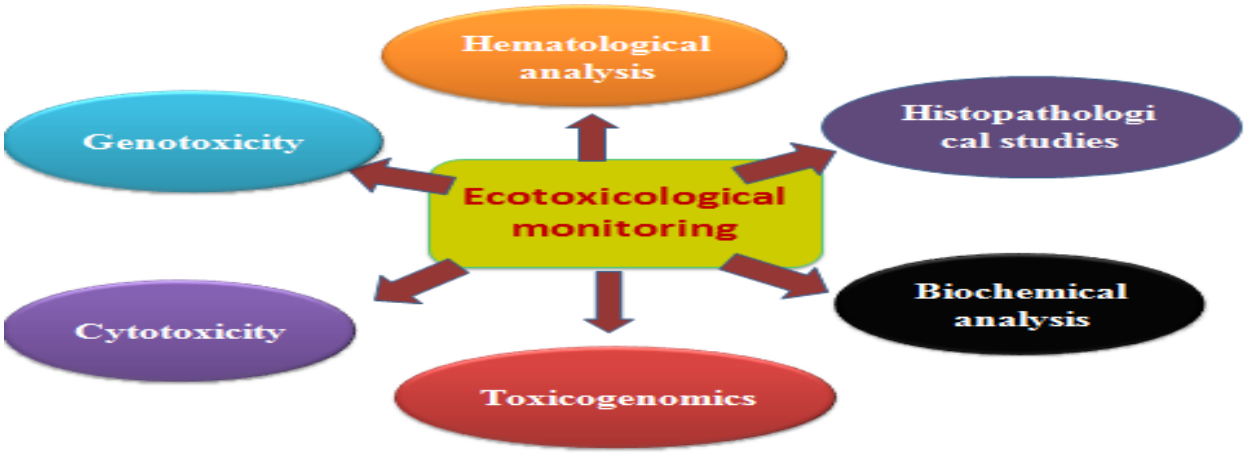
Table 3. Toxic substances (**Pesticides**) and their adverse effects in different fish species.

S. No.	Fish Fauna	Effect of Pesticides	Effect Organism
1	Catla catla	Endosulfan carbonyl	Signuficant histological alterration in gill.
2	Labeo rohita	Chlorophyrifos	Biochemical changes of total protein and glycogen observed.
3	Puntius punctatus	Endosulfan and Diazinon	Depletion in the activity of arginine and tryptophane showing the interaction of pesticides with cellular proteins.
4	Puntius punctatus	Endosulfan and Diazinon	Alteration of calcium content in the stomach after pesticides treatment.
5	Puntius punctatus	Rogon (dimithoate)	Abnormal behavior pattern in fish
6	Cyprinus carpio	Carbofuran 16ppm	Decrease in total erythrocyte count, total leucocytes count and hemoglobin count.
7	Cyprinus carpio & Puntius ticto	Aldrin, Dieldrin BHC and DDT	Bioaccumulation of chlorinated pesticides in fish tissue gill, liver muscle and kidney observed.
8	Mystus vittatus	Dimecron and Thiodon	Rate of food in take, absorption and metabolism decrease from the control value
9	Heteropneustes fossilis	Endosulfan .00075 ppm .00050ppm .000375ppm	Increased concentration, of toxicant showed the decrease in liver glycogen. Hepatic cells are damaged due to depletion of glycogen.
10	Heteropneustes fossilis	Dimecron	Significant decrease in Hb%, RBC number and O2 carrying capacity of blood.
11	Clarias batrachus	Carbaryl and Phorate	Cholesterol level in serum decreased during exposure period
12	Clarias batrachus	Phorate.27ppm	Physiological and histological disorder in testis and ovary of

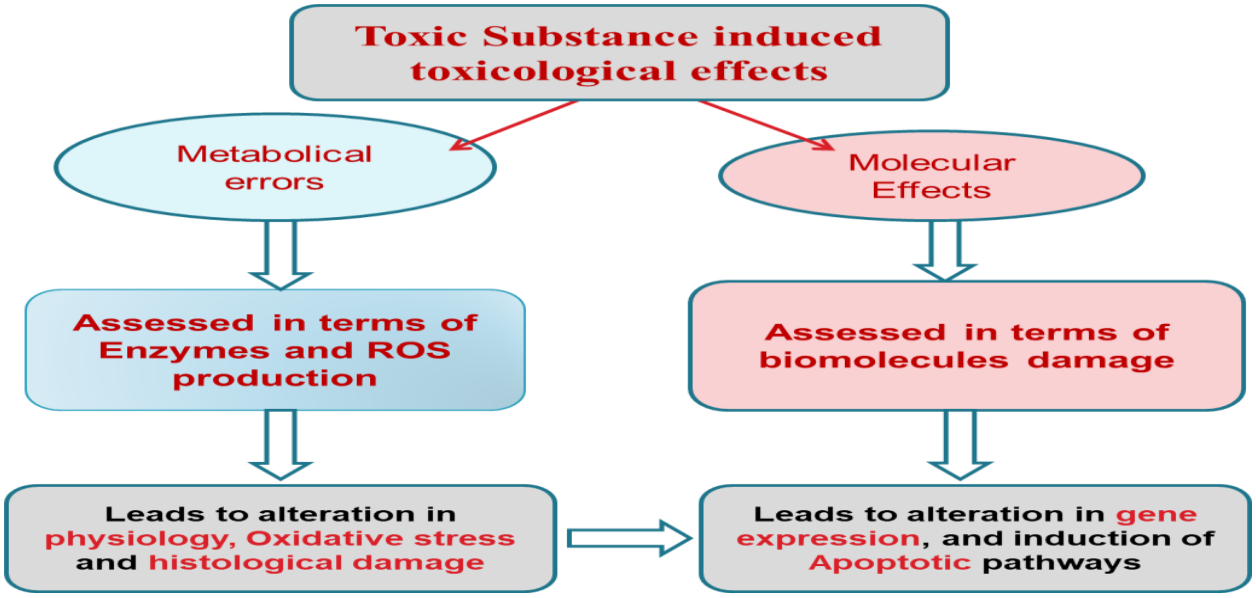
Different effects of toxic substances on life activities of fishes:



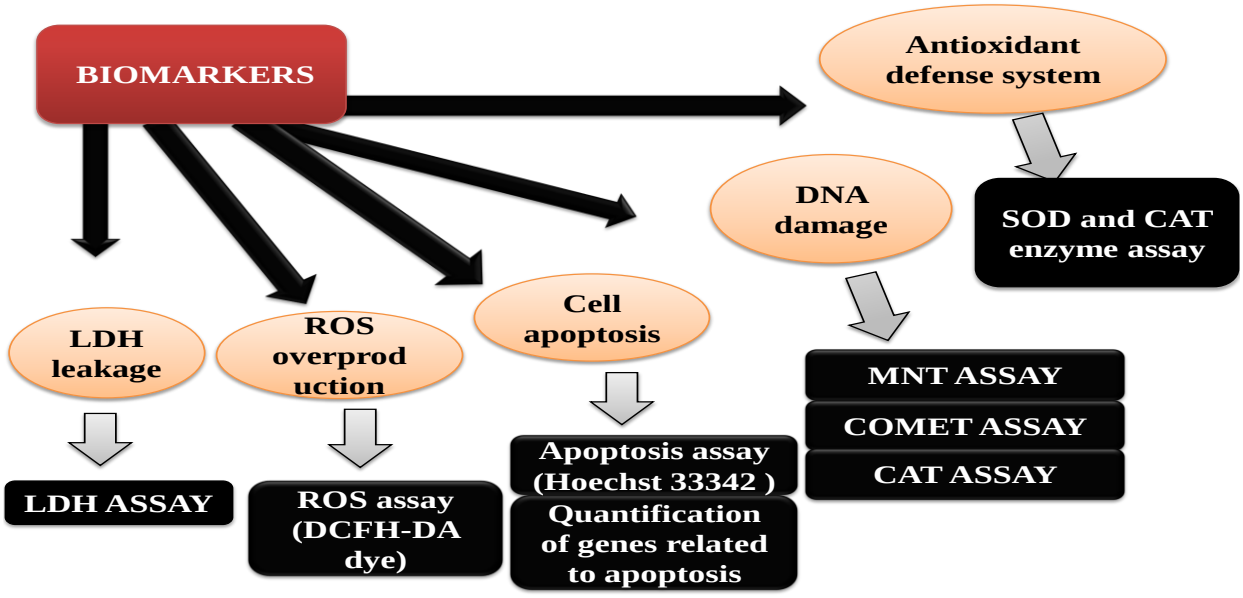
Types of toxicity: The toxicity broadly involves impact analysis of exposure of toxicants in fishes. Notable areas which are routinely employed ichthyo-toxicoloical research and aquatic bio-monitoring are displayed in the following picture.



Toxicological responses induced by different toxicants:



BIOMARKERS OF TOXICITY: A number of in vivo and in vitro biomarkers of chemical pollution are also found through some genotoxicity studies such as Micronucleus (MN), Chromosome Aberration (CA), and Single cell gel electrophoresis (SCGE) or Comet assay as reported by several authors (Farah et al., 2006; Kirsch-Volders et al., 2011; Kumar and Trivedi, 2015; Olive and Banáth, 2006; Podrimaj-Bytyqi et al., 2018; Xu et al., 2011). Moreover, OMET assay and MN assay are frequently used for the assessment of genotoxicity.



EXAMPLES OF BIOMARKERS OF GENOTOXICITY IN FISHES:

- **Micronuclei (MN):** Extracellular bodies that are arise in mitotic cells from acentric chromosomal fragments or chromosomes that lag behind and not integrated into the daughter nuclei. (Fenech and Morley, 1985; Schmid, 1982).At telophase, a nuclear envelope forms around the lagging chromosome and gradually, assume morphology of an interphase nucleus with exception that they are smaller than the main nuclei in the cell, hence the term micronuclei.

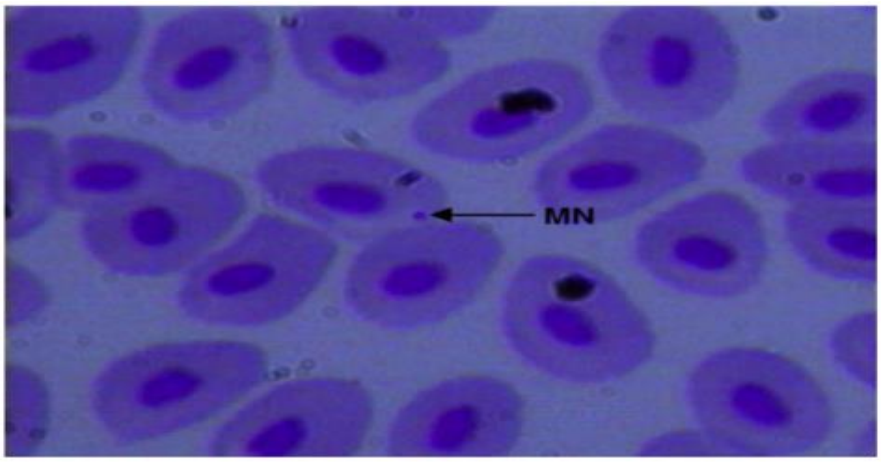


Fig. 2. Showing micronuclei induced by Hg(II) in peripheral erythrocytes of fish, *C. punctata*.

(Yadav and Trivedi, 2009)

- The **Comet assay or Single cell gel electrophoresis (SCGE)** is a widely used technique for measuring and analyzing DNA breakage in individual cells by performing single cell gel electrophoresis as a three layer procedure (Pandey et al., 2011; Tice et al., 2000).

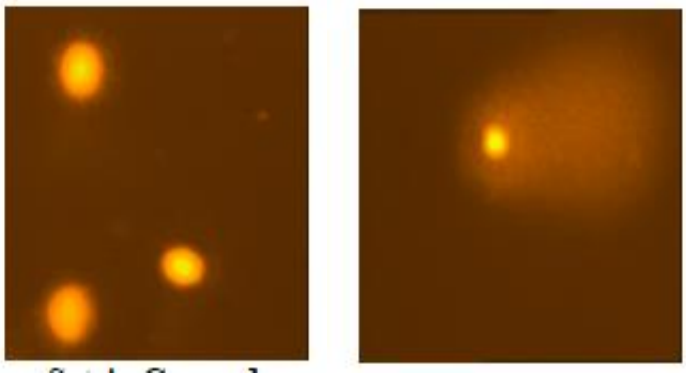


Fig. The SCGE shows a normal and comet tail containing cells, respectively in fish exposed to pesticide, Carbofuron (Pandey et al., 2011).

- **Gene expression and quantification**: Expression of p53 and other genes that are related to maintain the integrity of genetic material find ample application in studying the ill effects of water borne toxicants (Xenobiotics).

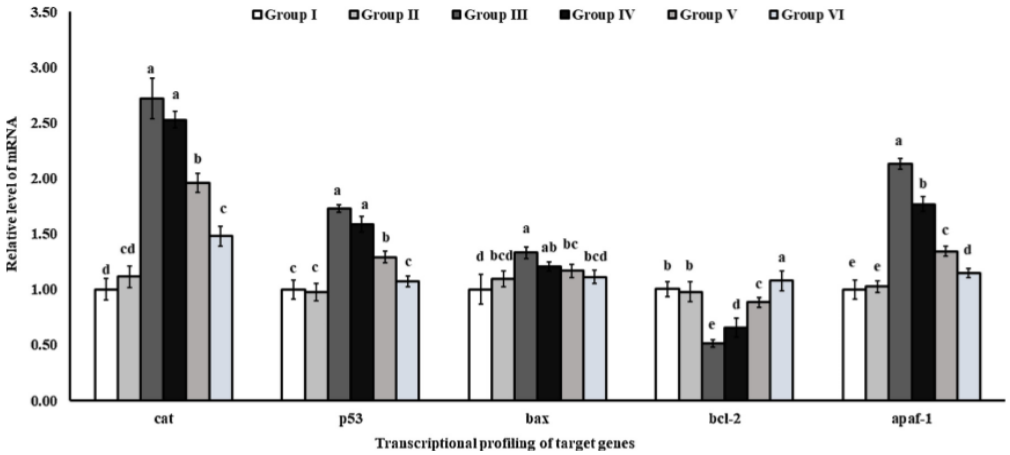


Fig. 5. Graph showing the relative transcript/mRNA level of target genes, determined by qRT-PCR, and normalized with *β-actin*. (Values expressed as mean ± S.E.M.; n = 9 fish in each group; values not sharing common letters (a–e) are significantly ($p < 0.05$) different; group I - control, group II - 3 mg/L of curcumin, group III - 7.89 mg/L of Cr^{6+}, group IV, V, VI - 7.89 mg/L of Cr^{6+} plus 1, 2, 3 mg/L of curcumin, respectively).

(Awasthi et al., 2019)

- **Biomarkers of apoptotic cells**: Apoptotic cells show nuclear condensation or compactness due to certain changes in nuclear morphology or chromatin structure (Candé et al., 2002). A small, rounded, brightly colored and compacted nucleus observed along with condensed chromatin in blood cells of Chromium (Cr6+ exposed fish, *Channa punctatus*, clearly depicts apoptosis (Awasthi et al.,2019).

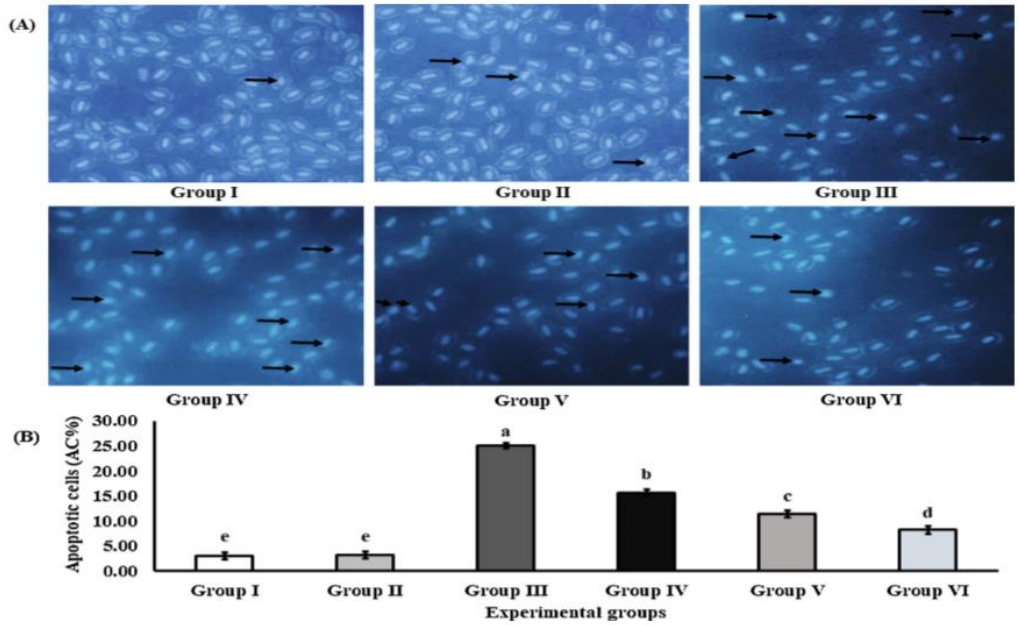
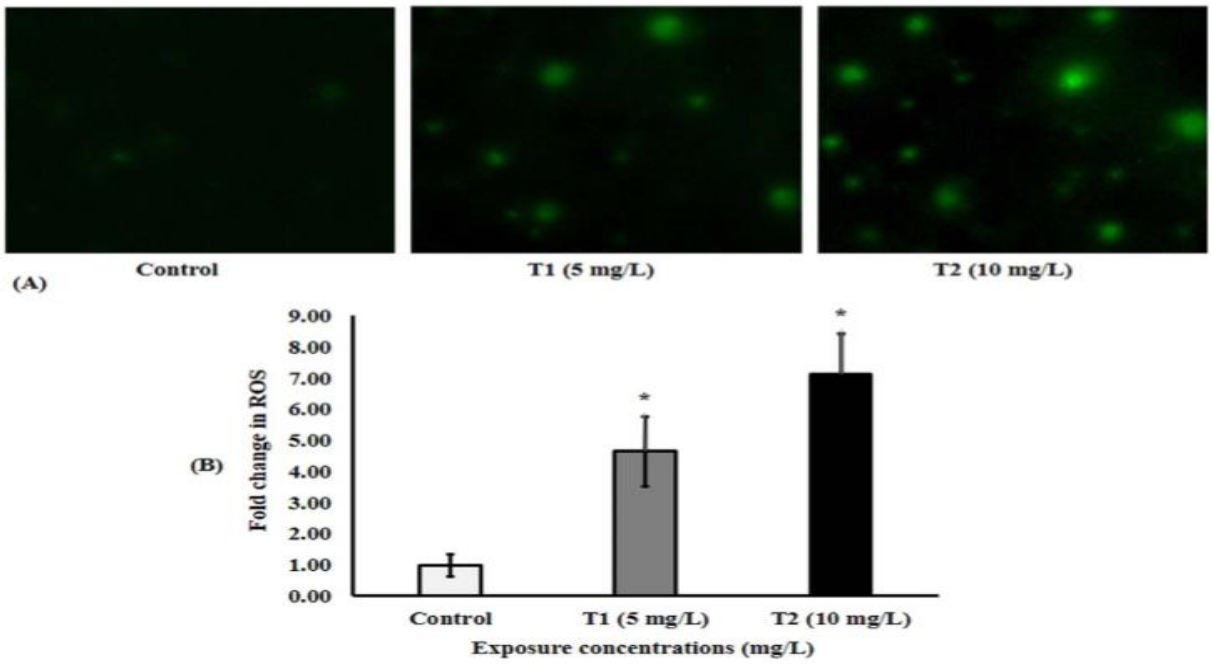


Fig. 4. (A) Microphotographs showing apoptotic cells (AC) in erythrocytes of *Channa punctatus* for experimental groups I, II, III, IV, V and VI after 45 d of exposure period. Blood cells were stained with Hoechst 33,342 (1.5 mg/mL) dye. Cr^{6+} induced nuclear shrinkage or condensation and fragmentation (arrows) and their progressive amelioration in group IV, V and VI in a dose-dependent manner is visible. **(B)** Graph showing the quantitated and integrated AC% in blood cells of test fish of all experimental groups. (Values are expressed as mean ± S.E.M.; n = 9 fish in each group; values not sharing common letters (a–e) are significantly ($p < 0.05$) different; group I - control, group II - 3 mg/L of curcumin, group III - 7.89 mg/L of Cr^{6+}, group IV, V, VI - 7.89 mg/L of Cr^{6+} plus 1, 2, 3 mg/L of curcumin, respectively).

- **ROS (Reactive Oxygen Species) estimation**: Cellular ROS levels can be measured in live cells by a technique that converts/ oxidizes 2',7' –dichlorofluorescin diacetate (DCFDA) to a fluorescencent dye 2', 7' –dichlorofluorescein (DCF). The fluorescence generated is directly proportional to the amount of oxidized DCFDA to DCF. Thus, ROS can be quantified with the help a software.



Microphotographs showing Zn2+ induced ROS level in blood cells of fish, *Channa punctatus*. (B) Values of fold change in ROS level using DCFH-DA dye for T1 (4.64±1.12-fold) and T2 (7.12±1.30fold) as compared to unexposed group (C) (Values are expressed as mean±S.E.M., n = 3 fishes in each replicate, *represents the significant ($p<0.05$) values in comparison to unexposed/control fishes).https://www.ncbi.nlm.nih.gov/pubmed/29304413.

ROS also leads histological damages in different tissues, e.g., Liver, of fish, *Channa punctatus*, https://link.springer.com/article/10.1007/s12595-017-0223-1

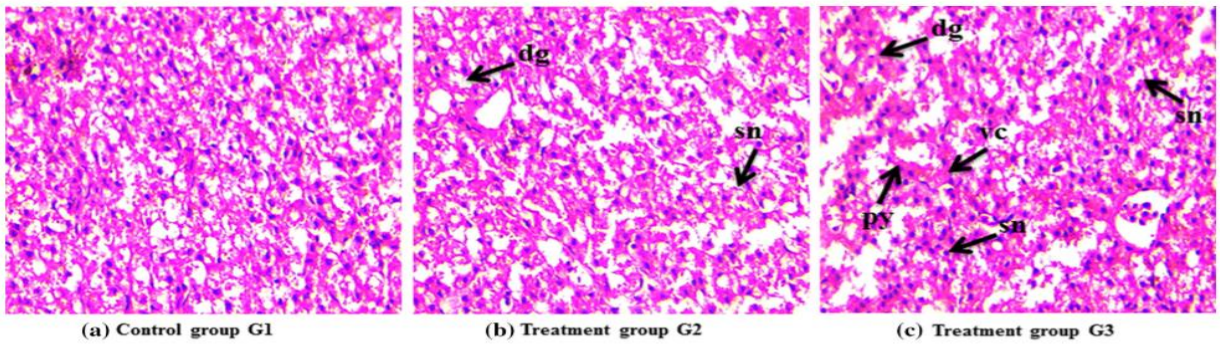


Fig. 3 Histology of liver tissue of fish *C. punctatus* in control (G1) and LAS treated (G2 and G3) after 96 h of exposure period through eosin-haematoxylin staining (**a**–**c**). The **a** shows the regular arrangement of hepatocytes in group G1 fish liver samples after 96 h while the **b** and **c** show hepatocytes impairments viz., degenerated hepatocytes: dg, sinusoidal space: sn, pyknotic nuclei: py, and increased vacuolization: vc in hepatocytes of samples of treatment group G2 and G3, respectively after 96 h of exposure period

Some more examples of toxic responses in fishes exposed to different toxicants (Heavy metls):

1. **Chromium – Induces ROS production, Oxidative stress, DNA damage and alteration in gene expression**
   https://www.sciencedirect.com/science/article/abs/pii/S0166445X18300432
2. **Arsenic- Induces DNA damage in fish**

   https://www.ncbi.nlm.nih.gov/pubmed/28573351
3. **Mercury and Copper induced toxicity in fishes:**

   https://www.ncbi.nlm.nih.gov/pubmed/19545645

Relevance/ significance of the Study :

For precise bio- monitoring of polluted aquatic habitats ichthyo-toxicological studies are of prime importance. Some new, rapid, and sensitive multiple biomarkers can be effectively employed in this new era of environmental monitoring. In addition to haematological and biochemical parameters of clinical significance, some of the routinely employed and validated methods can be applied for the assessment of genotoxicity in fishes. They include, CAT or Chromosomal aberration test as cytotoxic end markers, MNT or Micronucleus test as an index for chromosomal damage, Single cell gel electrophoresis (SCGE) or Comet assay for detecting alteration in DNA organization, activities of oxidative stress marker enzymes, SOD, CAT, and GR etc. for assessment of extent of Reactive Oxygen species (ROS) and expression and quantification of certain marker genes, *viz, p53* for DNA damage and repair, *bax, bcl2, apaf1, casp3* for apoptosis or programmed cell death, and *nox, gsr, sod,* and *cat* for assessment of oxidative stress in fishes surviving in compromised habitats..

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