IMMUNOTOXICITY OF INDUSTRIAL EFFLUENTS IN FIN FISH: AN ALTERNATIVE ANIMAL MODEL FOR IMMUNOTOXICOLOGICAL STUDIES

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Immunotoxicity

- Interaction of the Xenobiotics on immune system

  (Xenobiotic - A chemical that is foreign to the biosphere i.e. is not produced by a natural biological or abiotic source)

- Inhibit or depress immune function
  - Immunosuppression - Susceptibility to bacterial, viral and parasitic infection
  - Damage to primary and secondary lymphoid organs
- Elicitation of an immune response –

  Hypersensitivity & Autoimmune disease.

- ✓ The immune system represents the interface between an individual's health and the pathogens present in its environment.
- ✓ The immune system is exquisitely sensitive for assessing the toxic effects of chemicals of environmental concern.

Xenobiotics that elicit Immune Reactions

- Polyisocyanates - Toluene diisocyanate
- Acid Anhydrides - Trimellitic anhydride
- Metals & Metal Salts - Pt, Co, Ni, Cr
- Drugs – Penicillin
- Pesticides – Carbamates
- Polycyclic aromatic hydrocarbons & halogenated aromatic hydrocarbons – PCBs & PBBs
- Benzene
- Dibenzodioxin (TCDD – dioxin)
- Organophosphorous compounds, ozone, metals, organotin, cyclophosphamide

(Zeeman and Brindley, 1981; Dunier and Siwicki, 1993; Anderson and Zeeman, 1995; Luebke *et al*., 1997; Zelikoff *et al*., 2000; Bols *et al*., 2001; Rice, 2001; Burnett, 2005; Carlson and Zelikoff, 2008)

AQUATIC TOXICOLOGY

- Dissolved metals – minute amounts in the aquatic environment
- Through industry may be transported, concentrated, changed into other forms and are reintroduced into the aquatic system as contaminations.
- Fish are a fairly inexpensive protein-rich food that constitutes the sole protein source for many people. Unfortunately they are frequently exposed to many pollutants in the aquatic environment.
- Fish and their immune system may also represent an important scientific tool in the monitoring of environmental quality, particularly immunotoxic environmental pollution

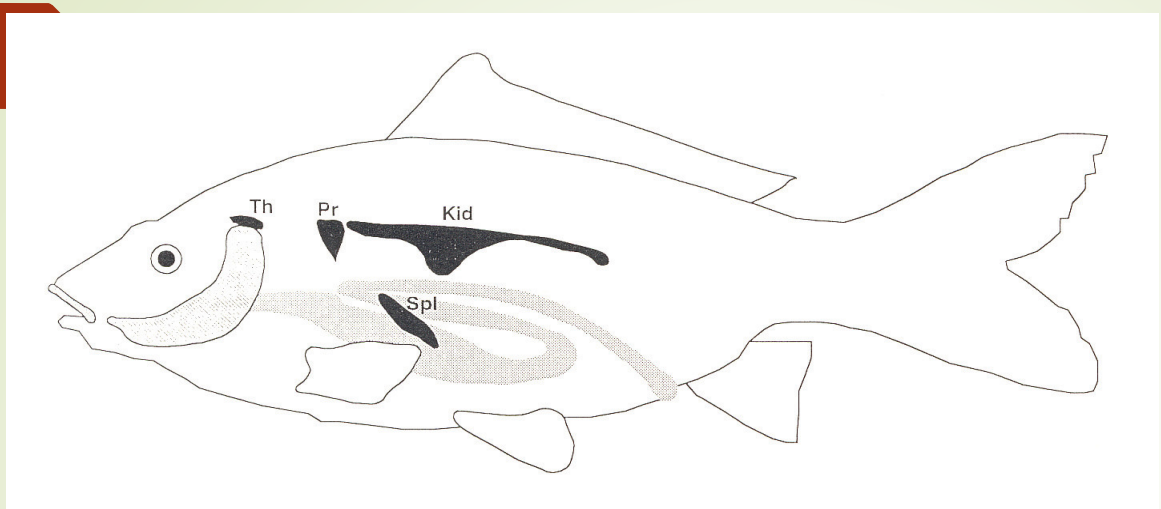
FISH IMMUNE SYSTEM

Fish are the first group of vertebrate animals with both innate and adaptive immune responses.

The immune system of fishes can be subdivided into broadly three categories which differ in the speed and specificity of response

1. First line of defence is external barriers separating the fish from its environment, i.e., the epithelia of skin, gills and alimentary canal
2. Inside the fish, the second immune category is formed by the innate immune system which enables a rapid response to invading pathogens.
3. The third line of immune defense is the adaptive or acquired immune system, a set of humoral and cellular components that enable a pathogen-specific response. Adaptive immune system of fish usually shows a rather slow response to infective pathogens, taking weeks instead of days as in mammals.

The immune system of teleost fishes



Humoral immunity (serum, mucus)	Cellular immunity (kidney, spleen, thymus, blood, peritoneum)		
Antibodies Complement Lysozyme Lectins C-reactive protein Interferons Transferrin Anti-proteases Proteases Eicosanoids Cytokines	Ig B	CD8 TCR CD4 Tc Th	MM G
	Circulating antibodies Antibody-forming cells Proliferation	Specific cytotoxicity Proliferation	Phagocytosis Respiratory burst Antigen presentation
	Cytotoxic activity (NCCs, NK, Tc), circulating leucocytes, infiltration, distribution, gene expression, cytokines…..		

The immune system of teleost fishes…

- The immune tissues are quite different since fish lack the bone marrow and lymphatic nodules.
- Pronephros (anterior/head-kidney) is the main lympho-haematopoietic tissue
- Thymus is the main tissue for T cells development and maturation.
- Spleen is the main secondary lymphoid tissue
- Other important site for the immune response is the mucosal associated-lymphoid tissue (MALT) – skin, gills and gut.
- The non-specific immune parameters are useful to determine the health status of fish and to evaluate the immunomodulatory substances for fish farming as markers for pollution and diseases resistances.

The immune system of teleost fishes…

- The humoral immune response is a compilation of proteins and glycoproteins
- The complement system, in plasma and mucus, shows classical, alternative and lectin activation pathways
- An important bacteriolytic enzyme is the lysozyme, mainly found in eggs, mucus, plasma and leucocytes
- There are also other innate immune factors such as acute phase proteins (C-reactive protein CRP), antimicrobial peptides, interferon (IFN), lectins, proteases, protease inhibitors or eicosanoids
- Ig are the major component of the adaptive humoral immune response.
- Fish have only one immunoglobulin isoform, the IgM - tetrameric instead of pentameric as it occurs in mammals.
- WBCs are functional equivalent to lymphocytes, granulocytes, macrophages and NK cells.
- Fish macrophages secrete a wide range of biologically active molecules including reactive oxygen species (ROS) including superoxide anion (O_{2-}), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) etc., which are involved in the bactericidal activity, during a phenomenon termed the respiratory burst (Secombes 1990).

QUESTIONS?

- How to assess immunotoxic effects?,
- What are the mechanisms leading to immunotoxicity?,
- What are the implications of the effects on immunocompetence and organism fitness?
- As the piscine immune cells and organs are closely associated with the blood system, and partly act as filtering system for the circulatory system, they are highly accessible to toxicants.
- Additionally, the immune system may be indirectly affected by toxicants via the neuro-endocrine system

Table 1. Examples of toxicant effects on immune parameters and disease susceptibility of teleost fishes *in vivo*.

Chemical agent	Fish species	Immune effect	Reference
Metals			
Cd, Hg	*Oncorhynchus mykiss*	Reduced respiratory burst activity of phagocytes	Sanchez-Dardon *et al.*, 1999
Organometals			
Organotin	*Oncorhynchus mykiss*	Reduced respiratory burst activity of leukocytes, enhanced pathogen susceptibility	Nakayama *et al.*, 2009
Persistent organic pollutants, POP			
Aroclor 1248	*Ameiurus nebulosus*	Impaired IgM production, enhanced pathogen susceptibility	Iwanowicz *et al.*, 2009
Polyaromatic hydrocarbons, PAH			
Benzo(a)pyrene	*Oryzias latipes*	Impaired lymphocyte proliferation, enhanced pathogen susceptibility	Carlson *et al.*, 2002; 2004
Pharmaceuticals			
Diclofenac	*Salmo trutta*	Inflammatory responses	Hoeger *et al.*, 2005
Pesticides			
Lindane	*Oncorhynchus mykiss*	Reduced oxidative burst, altered B cell function	Dunier *et al.*, 1994
Esfenvalerate	*Oncorhynchus tshawytscha*	Enhanced pathogen susceptibility	Clifford *et al.*, 2005
Endocrine disruptors			
17beta-estradiol	*Dicentrachus labrax*	Decreased plasma lysozyme and antibody titre	Thilagam *et al.*, 2009
Nonylphenol	*Ictalurus punctatus*	Altered phagocyte activity	Rice *et al.*, 1998
Mixtures			
Heavy oil	*Paralichthys oliveaceus*	Downregulation of virus defense-related genes; enhanced pathogen susceptibility	Nakayama, K. *et al.*, 2008; Song *et al.*, 2011

OBJECTIVE

- To investigate the effect of sub lethal concentrations of industrial effluents on the immunity of ***Cyprinus carpio*** in terms of
  - **nonspecific**
  - **specific**



Tannery industry

Coffee industry and

Electroplating industry

Effluent exposure

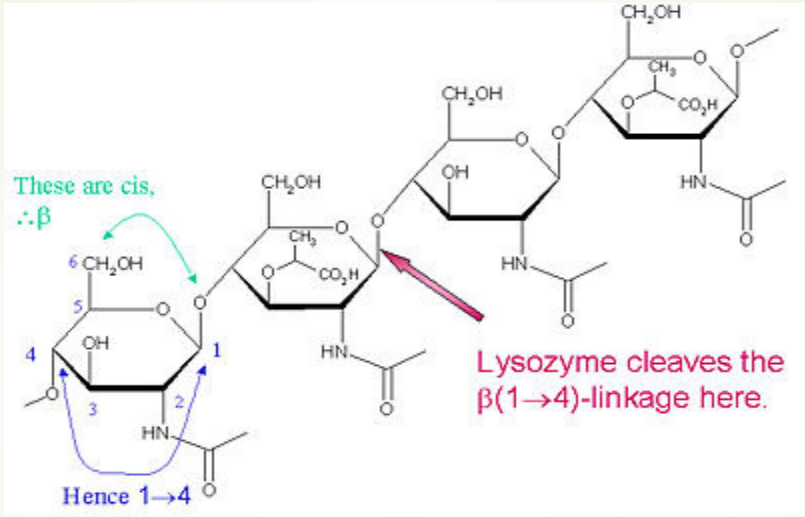
- 96 h LC_{50} -static bioassay method (Doudoroff *et al.,* 1951)
- Probit analysis (Finney, 1964).
- The 96 h LC_{50} of TE for *C.carpio* - 4.5% of TE.
- 0.0045%, 0.045% and 0.45%
- The 96 h LC_{50} of coffee mill effluent for *C.carpio* - 3% of effluent.
- 0.003%, 0.03% and 0.3%
- 96 h LC_{50} of electroplating industrial effluent for *C.carpio* - 0.128% of effluent.
- 0.004%, 0.007%, 0.03%, 0.010and 0.013%

Experimental Setup

- Two sets with four groups (6 fish/ Group) each
- SET I - non specific immune parameters - exposed to test concentrations for specified days
- SET II - specific immunity - exposed to test concentrations - immunized intraperitoneally with heat killed *Aeromonas hydrophila*.

SERUM LYSOZYME ACTIVITY

- **Cleaves β (1-4) between n-acetyl muramic acid and n-acetyl glucosamine present in the peptidoglycan cell wall of Gram (+) bacteria**

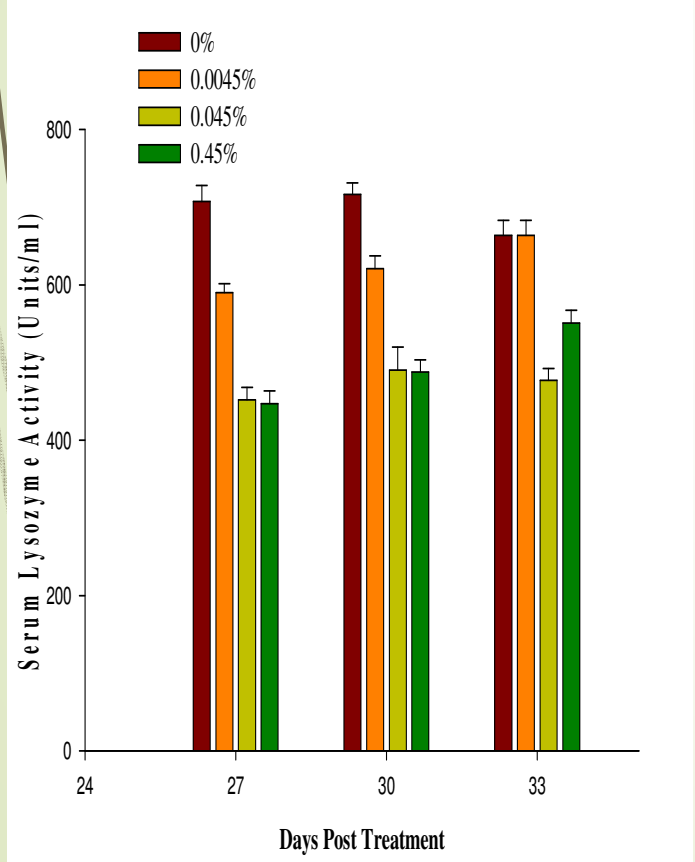


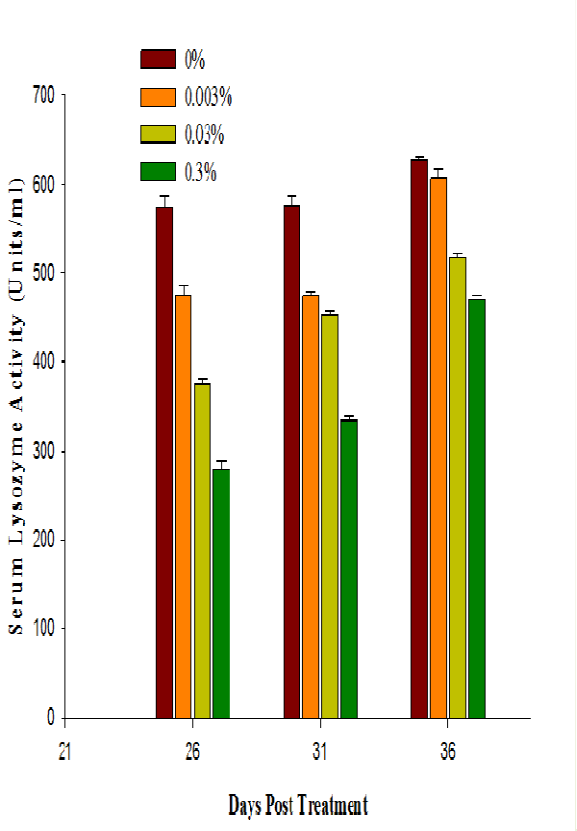
- **Serum lysozyme level was measured by a turbidimetric assay. The serum was incubated with *Micrococcus lysodeikticus* (substrate for lysozyme) and the reduction in absorbance at 490nm was calculated.**

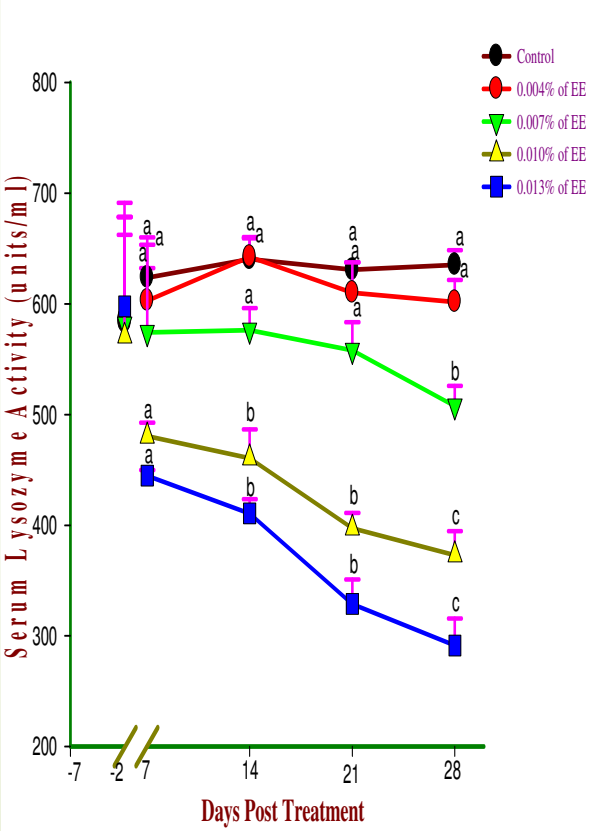
REF:

- Parry, R.M., Chandan, R.C., Shahani, K.M. (1965). A rapid and sensitive assay of muramidase. Proc. Soc. Exp. Biol. 119, 384–386.
- Hutchinson, T.H., Manning, M.J. (1996). Seasonal trends in serum lysozyme activity and total protein concentration in dap (*Limanda limanda* L.) sampled from Lyme Bay, UK Fish. Shellfish. Immunol. 6, 473–482.

Effect of chronic exposure to industrial effluent on the serum lysozyme activity in *Cyprinus carpio*

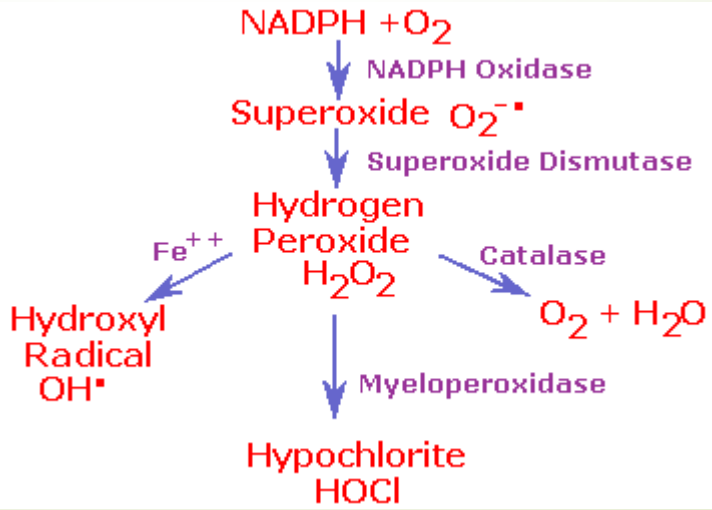






SERUM MYELOPEROXIDASE

MPO catalyses the oxidation of halide ions by H_2O_2 to form hypohalites, chloramines and singlet oxygen which are detrimental to pathogens



SERUM MYELOPEROXIDASE ACTIVITY

10µl of fish serum was added to 90 µl of HBSS (pH 7.3, without Ca^{2+} and Mg^{2+})

↓

35µl of TMB was added and incubated for 2 minutes

↓

35 µl of 2M H_2SO_4 was added to stop the reaction

↓

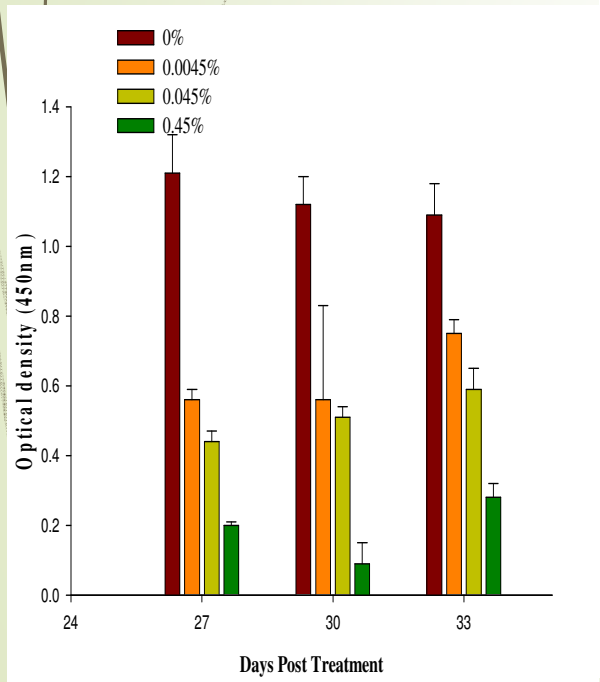
OD at 450 nm

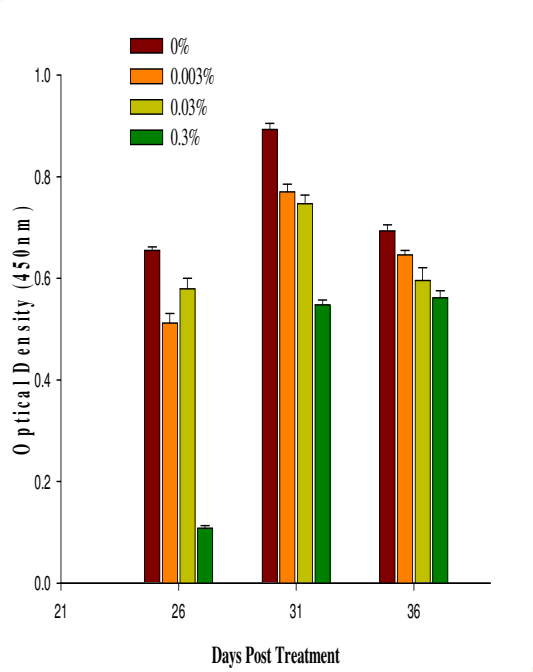
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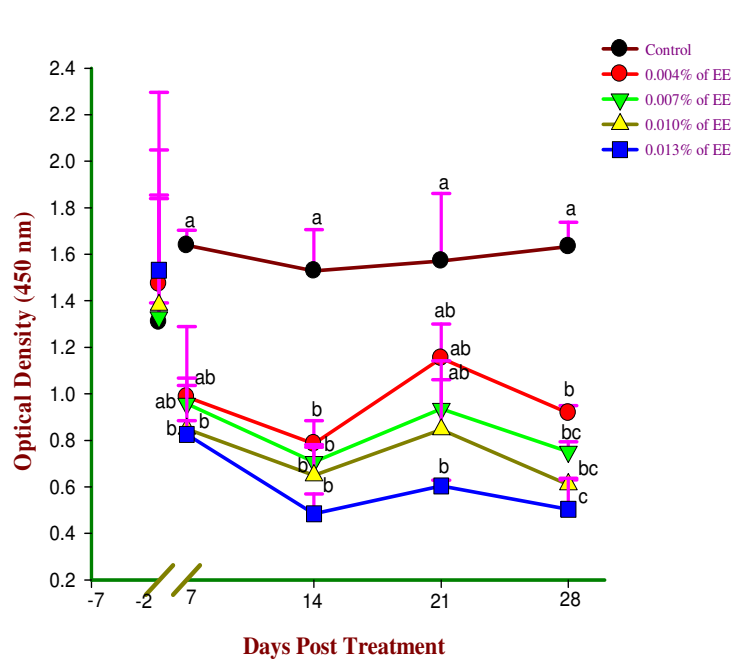
Quade, M.J.and Roth, J.A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology and Immunopathology 58, 239–248

Sahoo, P.K., Kumari J.and Misra, B.K. (2005).Nonspecific immune responses in juveniles of Indian major carps. Journal of Applied Ichthyology 21:151-155

Effect of chronic exposure to industrial effluent on the serum myeloperoxidase activity in *Cyprinus carpio*







SERUM ANTIPROTEASE ASSAY

Inhibition of trypsin is one way to measure antiprotease activity. Chromogenic substrate BAPNA is hydrolysed by trypsin amidase yielding yellow dye paranitroaniline which is measured photometrically

10µl of test sera was mixed with 20µl of Trypsin(1 mg/ml) in 0.01M Tris HCl. pH 8.2

↓

Incubated for 5 min.

↓

Add 500µl of 2 mM BAPNA and made up the volume to 1 ml with 0.1M Tris HCl

↓

Incubated for 25 min at room temperature

↓

Added 150µl of 30% Acetic acid.

↓

OD at 410nm.

The results are expressed in percent trypsin inhibition.

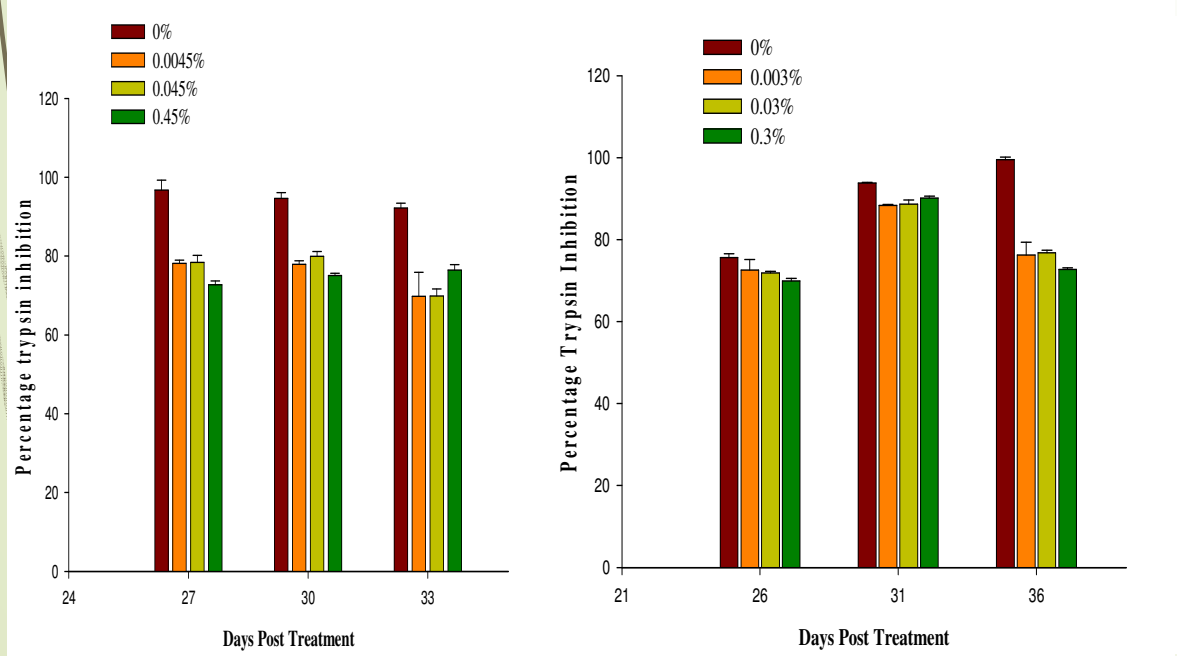
%Trypsin Inhibition = (A1 –A2 /A1) x 100

A1 = Control (Without serum); A2 = Sample

REF:

Bowden, T.J., Butler, I.R., Bricknell, I.R.and Ellis, A.E. (1997). Serum trypsin inhibitory activity in five species of farmed fish. Fish and Shellfish Immunology 7, 377-385

Effect of chronic exposure to industrial effluent on the serum anti-protease activity in *Cyprinus carpio*



ANTIBODY RESPONSE - BACTERIAL AGGLUTINATION ASSAY

25ml of antiserum was added to the first well of 96 well 'V' bottom microtitre plates

↓

Two fold serial dilutions were made using PBS

↓

25µl of heat- killed *A.hydrophila* cell suspension ($1x10^9$ cells/ml) pre-stained with crystal violet was added

↓

Shake well and incubate at 37°C overnight

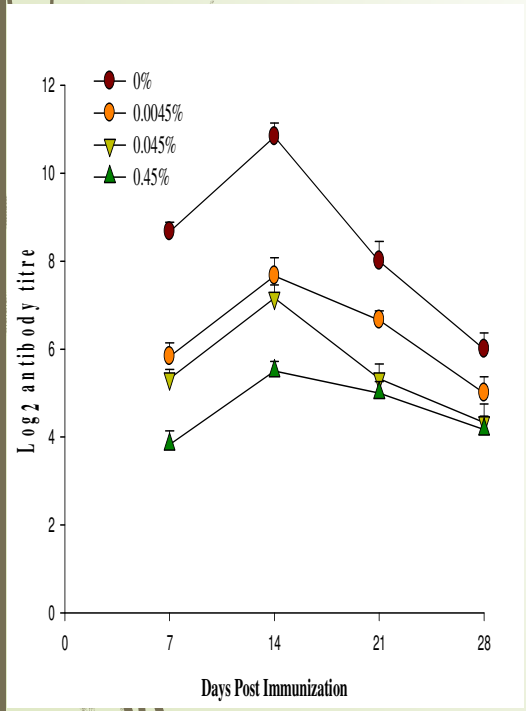
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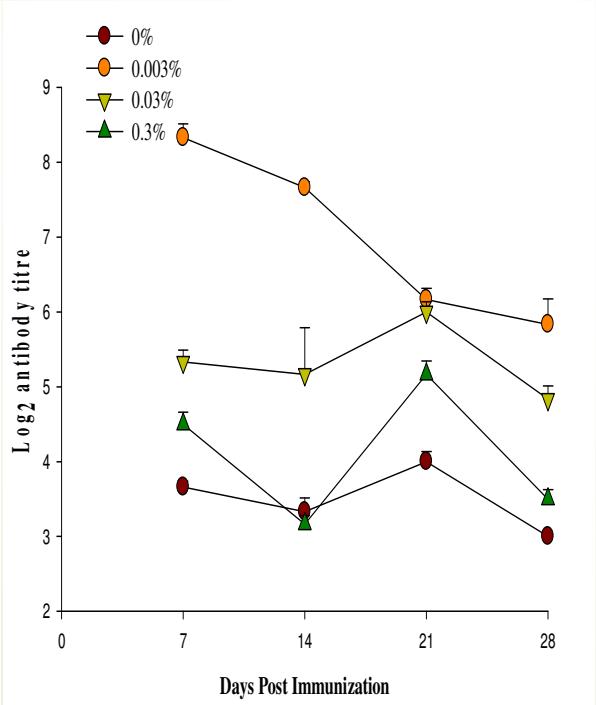
The highest dilution of serum sample that showed detectable macroscopic agglutination was recorded and expressed as $\log_2$ antibody titre of the serum.

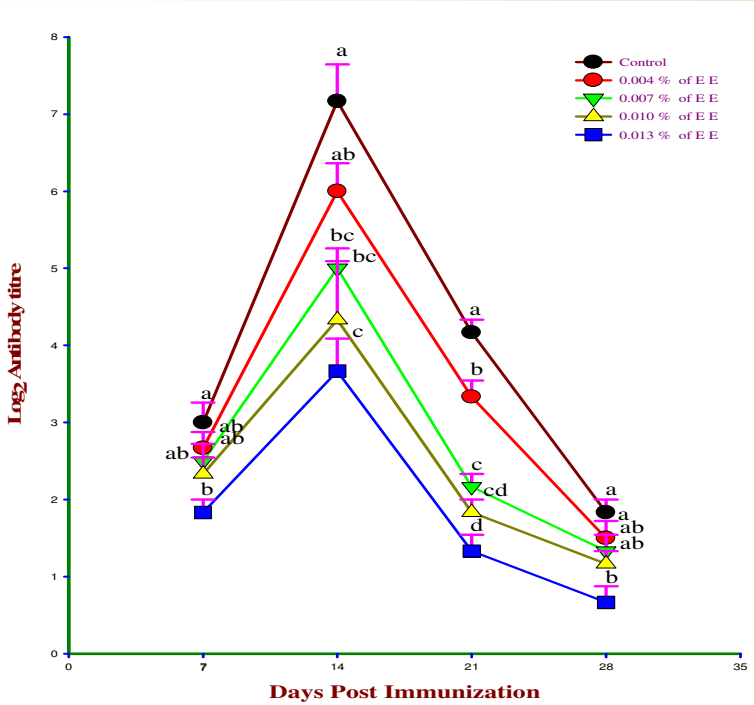
REF:

Karunasagar, I., Ali, A., Otta, S.K., Karunasagar, I. (1997). Immunisation with bacterial antigens: Infections with motile Aeromonads. Dev. Biol. Stand. 90, 135–141.

Effect of chronic exposure to industrial effluent on the antibody response to heat-killed *Aeromonas hydrophila* assayed by bacterial agglutination tested in *Cyprinus carpio*







Implications

- Assessment of the hazard of immunotoxic chemicals not only focus on the relationship between chemical exposure and the immune system, but it needs to take into consideration the complex functional properties and the ecological context of the immune system.
- The results of this study would emphasize the importance of integration of immunological assays into environmental monitoring with reference to industrial effluents.
- The immunotoxicity of industrial effluents may influence the ability of fish to defend against infectious diseases.

THANK YOU