INTRODUCTION

The worldwide distribution of toxic substances in the ecosystem is of major concern. Emission of anthropogenic pollutants in the environment has increased with progressive industrial development and a multitude of chemicals have been detected in environmental samples during the last decades (Stenstrom, 2013). Progress in science and technology coupled with population expansion and urbanization has led human to introduce a broad spectrum of substances both naturally occurring and synthetic into the environment. During the last three decades, there has been an expansion of various industries that produce various pollutants. These pollutants, directly and indirectly, affect the ecosystem (Smith *et al.*, 2013).

The U.S. Environmental Protection Agency (U.S. EPA) defines a pesticide as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest which is harmful to crop. A pesticide may also be described as any physical, chemical, or biological agent that will kill an undesirable crop or animal pest. The term pest includes harmful, destructive, or troublesome animals, plants, or microorganisms. Pesticide is a generic name for a variety of agents that are classified more specifically on the basis of the pattern of use and organism killed. In addition to the major agricultural classes that encompass insecticides, herbicides, and fungicides, one finds pest-control agents grouped as acaricides, larvacides, miticides, molluscides, pediculicides, rodenticides, scabicides, plus attractants (pheromones), defoliants, desiccants, plant growth regulators, and repellents.

There are different types of pollutants that affect both terrestrial and aquatic ecosystem. Terrestrial ecosystems play a key role in regulating diffuse contaminant transport to surface waters, particularly via the infiltration and retention of pollutants into soils. Throughout history, water has been used to wash away and dilute pollutants. Pollutant inputs have increased in recent decades and have degraded water quality of aquatic bodies (Chaudhry and

Malik 2017, Issaka and Ashraf 2016). Chemical pollutant entering into the aquatic system results in water pollution and is a major cause of global concern as it leads to the onset of numerous fatal diseases (Agarwal *et al.,* 2010). Many water pollutants are reported to act as toxic chemicals. Pesticides being one of them and is the most dangerous pollutant affecting aquatic system as it leads to water bodies from the fields as runoff after rain and flood (Kosygin *et al.,* 2007, Sarkar *et al.,* 2008 and Murthy *et al.,* 2013, Gilliom 2007, Rasmussen *et al*. 2011, Ansara-Ross *et al*., 2012).

The term "pesticide" is a composite term that includes all chemicals that are used to kill or control pests. In agriculture, this includes herbicides (weed), insecticides (insects), fungicides (fungi), nematocides (nematodes), and rodenticides (vertebrate poisons). Agricultural use of pesticides is a subset of the larger spectrum of industrial chemicals used in modern society. Modern agricultural practices reveal an increase of pesticides to meet the food demand of increasing population which results in contamination of the environment leading to the surfaced controversy of use and abuse of pesticide. The rampant use of these chemicals, under the saying "If little is good, a lot more will be better" has played a destruction with human and other life forms (Aktar *et al.,* 2009, Alexandratos and Bruinsma., 2012, EEA., 2013, Ullah *et al.,* 2015).

The use of pesticides has not been the same across the world due to the cost of the chemicals, but also due to the cost of manpower and the specific pests linked with each climatic and geographic region (Ramirez-Villegas *et al.,* 2012, Mayer, 2013 and Delcour *et al.,* 2015). According to FAO, the average application rates of pesticides per hectare of arable land have been computed and the highest average values have been found to be 6.5–60 kg/ha in Asia and in some countries of South America. In North America and West Europe, the use of herbicides is intensively applied in agriculture and in urban areas. However in the last decades; in Asia, the use of herbicides has remained low in contrast to the use of insecticides in other countries (Carvalho, 2017). The American Chemical Society database indicates that there were some 13 million chemicals identified in 2000 with some 25000 new compounds being added annually (Widener, 2017). In the Great Lakes of North America, for example, the International Joint Commission has estimated that there were more than 200 chemicals of concern in water and sediments of the Great Lakes ecosystem. Because the environmental burden of toxic chemicals includes both agriculture and non-agricultural compounds, it is difficult to separate the ecological and human health effects of pesticides from those of industrial compounds that are intentionally or accidentally released into the environment, there is overwhelming evidence that agricultural use of pesticides has a major impact on water quality and leads to serious environmental consequences (Pier *et al.*, 2001 and Parris, 2011).

Pesticides pollute water sources through direct application in aquatic systems or through indirect sources such as by erosion from agricultural lands and agricultural wastewater infiltration, which eventually enter in the aquatic ecosystem (Dutta and Arends, 2003). According to EPA report (2015), more than 50% of water pollution of streams and rivers occurs due to leaching and mixing of pesticides from agriculture practices (Agrawal *et al.*, 2010). Aquatic environment is not the actual target for pesticide, but the widespread use of them has led to serious problems including toxic residues in grass and toxicity of non-target organisms such as mammals, birds and fish (Saeed *et al.*, 2012; Shankar *et al.*, 2013).

According to Echobichon 2001, pesticides used these days have generally a higher selectivity and specificity towards the target-organisms and are more readily degraded in comparison to the pesticides used some decades ago, e.g. DDT, dieldrin and HCH. However, due to their inherent toxicity to certain non-target organisms, the environmental fate of modern pesticides is of great concern. Various studies have shown that streams with an agricultural catchment area are susceptible to brief pesticide inputs (Moss, 2008, Uppsala 2013). Many taxa of freshwater macroinvertebrates are confined to running waters, and Chironomidae (midges), Odonata (dragonflies and damselflies), Ephemoptera (mayflies) and Plecoptera (stoneflies) are believed to have evolved in cool running waters, biological effects of pesticides have been reported for different macro invertebrates (Berenzen *et al.*, 2005, Beketov *et al.* 2008, Weston and Lydy 2014). Benthic algae are usually the major primary producers in streams and fill an important ecosystem function as they provide food for higher trophic levels. Diatoms constitute the base of aquatic food webs; effects on algae will indirectly induce effects on higher trophic levels (Fleeger *et al*., 2003). Many studies on algae have shown that long-term and repeated exposure to pesticides induces tolerance (Berard *et al*., 2002, Downing *et al.,* 2008, Fairchild *et al.,* 2009,) which could be considered a long-term effect (Groner and Relyea, 2011 and Halstead *et al.,* 2014). Microorganisms donate significantly to primary production, nutrient cycling, and decay in anestuarine ecosystem; therefore, harmful effects of pesticides on microbial species may have consequent impacts on higher trophic levels. Pesticides may affect estuarine microorganisms via spills, runoff and drift (DeLorenzo *et al*., 2001, Widenfalk *et al*., 2004, Virag *et al*, 2007, Staley *et al.*, 2015).

Agriculture disrupts all freshwater systems hugely from their pristine states. Both agricultural and freshwater systems are complex and the relationships between them make a mesh of many dimensions. Not least there are many sorts of agricultural system and a plethora of natural waters and communities. The entire land surface, much of which is agricultural, forms the catchment area for one or other river system and almost anything that happens on the catchment has an effect on the freshwaters (Moss, 2008). At the individual level, pesticide interference can lead to reduced survival, growth or reproduction of an organism. Reduction in primary productivity or prey availability can secondarily lead to changes along in the food chain. At the community level, there could be changes in species number or diversity, reduced number of sensitive species and shifts of dominating species and thereby altering the habitat structure (EPA 2011).

Modern agriculture practices reveal an increase in the use of pesticides to meet the food demand of increasing population which results in contamination of the environment. In India, crop production increased to 100%, but the cropping area has increased marginally by 20% (Agrawal *et al.,* 2010). Pesticides have played a major role in achieving the maximum crop production but maximum usage and accumulation of pesticide residues are highly detrimental to the aquatic and other ecosystems (Goldar and Banerjee, 2004). Pesticide residues in drinking water have become a major challenge over the last few years. One of the terrifying effects of pesticide contamination of groundwater came to light when pesticide residues were found in packaged natural mineral water and popular cold drinks (CSE, 2002 and Gupta *et al.,* 2009). Most of the Indian rivers and their tributaries viz., Ganges, Yamuna, Godavari, Krishna, Sone, Cauvery Damodar and the Brahmaputra are reported to be grossly polluted due to the discharge of untreated sewage disposal and industrial effluents directly into the rivers. These wastes usually contain a wide variety of organic and inorganic pollutants including solvents, oils, grease, plastics, plasticizers, phenols, heavy metals, pesticides and suspended solids which are not easily susceptible to degradation. The indiscriminate dumping and release of wastes containing hazardous substances into rivers become a potential source of stress to biotic community. In addition to domestic and industrial discharge into the rivers, there occurs a continues surface runoff from agricultural areas as reported by Singh and Singh (2008) where they have found pesticide accumulation in fish. Another shocking incident has also been reported in the Chambal Sanctuary, where there was a sudden death of crocodiles. Experts agree that tilapia, an exotic fish species, could be the possible carrier of toxins and consumption of this species by crocodiles may have led to their death (Daniel, 2006 and Hosdodde, 2014).

Bio-accumulation of pesticides in aquatic species is increasing alarmingly, posing a threat to aquatic life. The process of bioaccumulation starts when pesticides applied to the agricultural land runoff during storms into the river, streams and eventually the ocean. The pesticides become parts of the water column and the fish ingest the pesticides, usually through their gills and sometimes through their scales. The pesticides then enter into their organs and fat tissue which are sequestered and gets stored in them. These accumulated pesticides gets biomagnified up the food chain as big fish eat small fish and eventually as humans eat the fish as it is one of the major sources of essential proteins for human beings. A variety of fish species showed uptake and accumulation of pesticides (Csillik *et al.,* 2000; Dhasarathan *et al.,* 2000, Moore and Waring, 2001, Pazhanisamy and Indra, 2007, David *et al.*, 2009,Wasim *et al.,* 2009, Thenmozhi *et al.*, 2011,Bhandare *et al.*, 2011,Sanjoy and Rita., 2012, Banaee *et al.*, 2013, Shankar *et al.*, 2013, Parikh *et al.*, 2013, Desai *et al.*, 2014, Sadekarpawar *et al.*, 2015, Sabra *et al.,* 2015 and Jiang *et al.,* 2016). Pesticides have been found to be highly toxic not only to fishes but also to fish food organisms, thus threatening the life of the fish (Sindun and John Thomas 2002, Dutta and Meijer, 2003, Satayavani *et al.*, 2011, Murthy *et al.*, 2013, Smalling *et al.*, 2013).

Most of the studies till date have focused on various different insecticides such as Neonicotinoids, which has been well explored by (Tomizawa and Casida 2005, Tennekes, 2010, Jeschke *et al.*, 2011, Parikh *et al.,* 2013, Gibbons *et al.,* 2015, Bayo *et al.,* 2016 and Tyor and Harkrishan, 2016), fungicides (Juerrgensen *et al.,* 2000, Åkerblom, 2004, Knauer *et al.*, 2007, Mellish, 2013 and Parikh *et al.,* 2014) and fertilizers/plant nutrient (Palanivelu *et al.,* 2005; Yaro *et al.,* 2005, Bobmanuel *et al.,* 2006, Biello, 2008, Ofojekwu *et al.,* 2008 a and b; Helfrich, 2009, Capkin *et al.*, 2010; Nowell *et al,* 2014., Parikh *et al.*, 2015, Akoto *et al*., 2016). Comparatively, there are few studies on the toxic effect of herbicides on fishes.

Herbicide is a type of pesticide which are widely used in agriculture, industry, and urban areas to control weeds. They are considered to be plant poisons and are relatively easy to apply and they are the only available practical method to control weed in terrestrial as well as aquatic habitat. They have made a large contribution in reducing the workload of crop production. While herbicides with various modes of action are currently used; sulfonylurea herbicides which were developed in late 1970s, are used extensively widespread use worldwide, and they hold the central position in weed control globally as paddy rice herbicides. Numerous commercial formulations containing different herbicides (glyphosate, paraquat, sulfonylurea etc) have become popular around the world due to their effective action and low toxicity to mammals. However; they have proven to be harmful to the environment, particularly weed-infested waters with herbicides have proved to be toxic to aquatic life (Rzymski *et al.*, 2013). The chemical era for the development of herbicide was an accident way back during World War II when scientists from the United States and England initiated research on plant growth regulators such 2,4 D and dinitro compound (Rao, 2000). Herbicide can be selective or non-selective. Selective herbicide suppresses certain plants without affecting the growth of other plant species. Selectivity is due to translocation, differential absorption, physical (morphological) or physiological differences between plant species (eg. 2,4-D, mecoprop, dicamba). Non selective herbicides are not specific in their action and kill all plant material with which they come into contact. They are used to clear industrial sites, waste ground, railways and railway embankments (eg. Paraquat, Glufosinate, Glyphosate).

Herbicide can also be classified depending upon their mode of action, relative time of application and chemical composition. However, classification based on site of action of herbicide is comparatively better as herbicide resistance management can be handled more properly and effectively. The International Herbicide Resistance Action Committee (HRAC) has published a classification system based on letters for each group and later on Mallory-Smith and Retzinger (2003) updated the classification; adequate changes were done to align this classification in accordance with classification published by HRAC. Herbicides generally target essential metabolic processes in plants e.g. photosynthesis, mitosis or amino acid biosynthesis. These processes are common in both crops and weeds.

Amino Acid Synthesis Inhibitors are broad spectrum of selectivity is used at low rates as soil-application and post-emergence treatment in a variety of crops. The amino acid derivatives are relatively non-selective post-emergence treatments. These herbicides are readily absorbed by both roots and foliage and translocated in both the xylem and phloem. They can be used as a direct application to soil or as a foliar treatment. Glyphosate is the example of this group of herbicides. Glyphosate is non-selective and is very tightly bound to the soil so no root uptake occurs. Applications must be made to plant foliage. Translocation occurs out of leaves to all plant parts including underground storage organs of perennial weeds (Dekker, 1995). Translocation is greatest when plants are actively growing. These group of herbicides inhibits the enzyme EPSP synthase, a key enzyme in shikimate pathway, which converts shikimate-3 phosphate to 5-enol pyruvylshikimate -3-phosphate and preventing the biosynthesis of aromatic amino acids like- tryptophan, tyrosine and phenylalanine. Branch chain Amino Acid Biosynthesis Inhibitors is a group of herbicides which are also known as ALS or AHAS inhibitors. Generally, these herbicides are absorbed in plant roots and foliage and are readily translocated in the xylem and phloem (Anderson, 2002). ALS interferes with acetolactate synthase (ALS), an enzyme involved in the synthesis of branched-chain amino acids, specifically valine, leucine and isoleucine. These amino acids are necessary for protein synthesis and plant growth. Post application of herbicide symptoms include plant stunting, chlorosis (yellowing) and tissue necrosis (death) which are evident within 1 to 4 weeks, depending upon the plant species and environmental conditions.

According to HRAC; considering the site of action, 2(B) group includes Inhibitors of Acetoacetate Synthase (ALS), also called acetohydroxyacid synthase (AHAS),comprises many chemical families- Imidazolinone, Pyrimidinylthiobenzoate, Sulfonylaminocarbonyltriazolinone, Triazolopyrimidine and Sulfonylurea; thus these five classes of ALS inhibitors have been commercialized (Vencill 2002, Mallory-Smith and Retzinger 2003). Almost 30 commercial herbicides have been identified from sulfonylureas. Each class of herbicide is unique as they bind to ALS at different sites which are overlapping. It has been postulated that ALS specific herbicides bind to the enzyme at the entry site for ALS substrate or substrate access channel (Pang *et al*., 2003). This inhibits the activity of the enzyme and leads to the deficiency of branched chain amino acids. Finally, there is death of susceptible species of plant due to starvation for branched chain amino acids (Tan *et al.,* 2005).

Many herbicides have undergone several toxicity testing to evaluate effects on a nontarget organism such as fish (Masser *et al.,* 2013, Nwani *et al.,* 2010). The herbicide that is toxic to the early life stages of fish has been reported in *Clariasgarie pinus* and scientists have opined that herbicide toxicity can lead to a decline in their populations (Ayoola, 2008 a and b, Ayanda and Egbamuno, 2012). Acute and chronic exposure to the sublethal concentration of paraquat commercial known by the name roundup of Nile Tilapia (*Oreochromis niloticus*) has been well studied by Jiraungkoorskul *et al.,* 2002, 2003 and Lushchak *et al.,* 2009. Atrazine herbicide toxicity has been well explored on *Clariaspunctaatus* (Nwani *et al.,* 2010); on Nile Tilapia (*Oreochromis niloticus)* (Peebua *et al.,* 2007 and De Ventura *et al.,* 2008); on Zebra fish embryo (Weigand *et al.,* (2001) as well as on blue gill sun fish (*Lepomis macrochirus*) by Elia *et al.,* 2002.

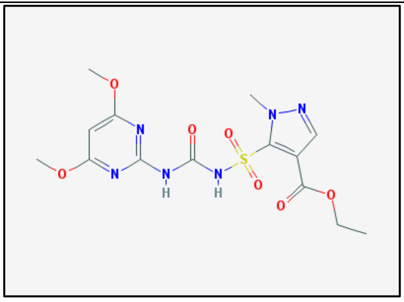
Glyphosate is a highly water soluble substance and its toxicity is based on the compound used for its formulation. The strong risk linked with glyphosate is they contaminate surface water bodies and ground water in the area of applications which constitutes a serious threat to the ecological balance of the region affected because of their toxic potential to alter food chain (Mallory and Ratzinger, 2003). The toxicity and its ecological risk have been analysed in gold fish by Lushchak *et al.,* (2009) and Cavas and Konen, (2007). Acute and chronic toxicity of two commercial formulations of herbicide having glyphosate as an active ingredient has been studied in *Danio rerio* and *Poecilia reticuleta* by Jofré *et al.,* (2013). Short term exposure to paraquat at sublethal concentration has induced a biochemical, physiological and histological alteration in *Clarias gariepinus* (Ladipo *et al.,* 2011).

Sulfonylurea herbicides are an important class of herbicides used worldwide for controlling weeds in all major agronomic crops. They were discovered by Levitt with DuPont and have strong points such as (i) low toxicity for untargeted organisms (ii) lower application rate than conventional herbicides, (iii) broad herbicidal spectrum ranging from broadleaf weeds to grasses, and (iv) safety to various crops that can be impacted/ imparted by structural modifications. It has come to hold a foremost position among selective herbicides. It consists of three basic molecular components: an aryl group, a sulfonylurea bridge and a nitrogen-containing heterocycle. Chemical variation among these components leads to the distinctive herbicidal activity, crop selectivity and soil activity of each of the commercialized member of this family. They are absorbed and transported through weed's root, leaves and stem and they act systemically to control weed both before and after emergence from the soil. The features of sulfonylureas make it possible to develop one-shot herbicides which can control weeds in paddy fields with a single treatment. These herbicides save much labour in the cultivation of paddy rice and are used in almost all paddy fields (Fig I).

Fig I: Paddy Crop Field where herbicide PE is sprayed.

Among sulfonylurea products, Pyrazosulfuron-ethyl (PE) herbicide is widely used for selective post-emergence control of annual and perennial grasses and broadleaved weeds in cereals. The IUPAC name of PE is Ethyl 5-[(4,6- dimethoxypyrimidin -2-ylcarbamoyl) sulfamoyl]-1-methylpyrazole-4-carboxylate. Structurally, it consists of a pyrazole ring linked to a pyrimidine through a sulfonylurea bridge. It is different from other sulfonylurea herbicides in the substitutions on the pyrazolexring; it does not include a triazinic and pyridinic ring (Sarmah and Sabadie, 2002). Therefore, common degradation pathways occurring on sulfonylureas, such as O- and N-dealkylation of the group on the triazine ring or triazine ring opening to form a triuret does not take place in PE. Information pertaining to the metabolism or transformation of this novel herbicide in the environment is poorly documented (Zheng *et al.*, 2008) (Table I).

Table I: Pyrazosulfuron-Ethyl (PE) compound details

Pesticide type	Herbicide
Substance group	Sulfonylurea
Mode of action	Broad-spectrum activity absorbed by roots and translocated throughout plant by controlling the synthesis of amino acids. Inhibits plant amino acid synthesis - acetohydroxyacid synthase AHAS
CAS RN	93697-74-6
Chemical formula	$C_{14}H_{18}N_6O_7S$
Molecular Structure	
IUPAC name	Ethyl 5-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-1-methylpyrazole-4-carboxylate
Manufacturers and suppliers of products using this active now or historically	Nissan Chemicals King Tech Corp
Example products using this active	Sirius 70 WG Sideral **Sathi (10% WP-by United Phosphorus Limited** Act Sunwell

Source: e- pesticide manual, 5.1 version, 15th edition

The occurrence of sulfonylurea herbicides in aquatic environments is receiving public attention (Battaglin *et al.*, 2000). Residues of sulfonylureas have been detected in surface water and groundwater due to runoff and leaching after their application (Okamoto *et al.*, 2001). Due to their high herbicidal activity, some crops (e.g., legumes and pastures) are highly sensitive to trace-level residues of sulfonylurea herbicides in soils. An increased understanding of the environmental fate and behaviour of sulfonylureas is imperative to reduce their potential negative effects on agronomic systems.

PE is widely used in rice crops in India and is currently recommended for use on some relevant crops in over 30 countries (Singh *et al.,* 2012a and 2012b; Pal *et al.,* 2012). Due its widespread use, it has become a potential water pollutant and presents an environmental risk, especially for aquatic organisms, owing to its fairly high water solubility which results in its high mobility and environmental detection in surface and groundwater (Battaglin *et al.,* 2000). The dissipation pattern of herbicide Pyrazosulfuron ethyl has been studied in different soil types and has explained residual behavioural of new generation herbicide in agro-ecosystem (Rao, 2002, Mukherjee *et al.,* 2006). The photolysis studies on pyrazosulfuron-ethyl have proven that its photodegradation is dependent on the pH and irradiation wavelengths of aqueous solution (Wang *et al.,* 2013, Vulliet *et al.,* 2002 and 2004, Fidente *et al.,* 2006,). Phytotoxicity of chlorsulfuron, sulfometuron-methyl and metsulfuron-methyl has been reported for higher plants (Sabater and Carrasco, 1997). Toxicity of triasulfuron on aquatic organisms has been reported earlier (Baghfalaki *et al.,* 2012***).*** At high concentration, sulfonylurea are known to reduce the survival, growth and reproduction of fish, and produce many visible effects on fish (Rahman *et al.,* 2002). Pyrazosulfuron ethyl (PE) is relatively newpost emergence sulfonylurea herbicides (Angiras and Kumar 2005 and Rajkhowa *et al.,* 2006).

Studies till date in our lab has focused on the toxic effects of a variety of agrochemicals including insecticide- Dimethoate, Imidaclorpid; fungicide -cymoxanil and Mancozeb as well as plant nutrient mixture on freshwater fishes.(Fig II).

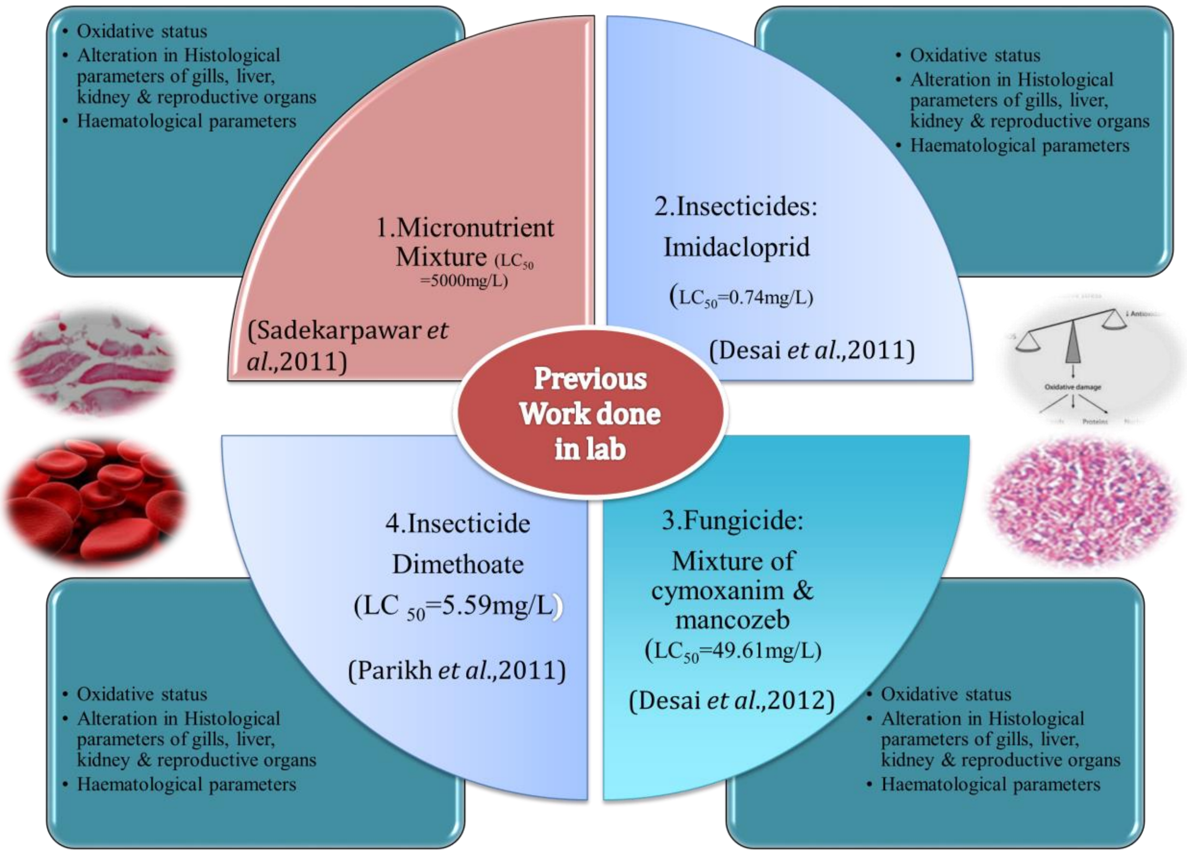


Fig II: Summary of previous work done in the lab

However, approaches to the selective action of pyrazosulfuron-ethyl, a herbicide in physiological and biochemical aspects have been little studied, as evidenced by the foregoing literature and thus there is an overall lacunae in the information regarding the toxic effect of herbicides in general and pyrazonsulfuron ethyl in particular to our knowledge. Thus, we hypothesize that the herbicide upon leaching to nearby freshwater ecosystem can possibly enter into the food chain which can result into decrease in the dynamicity of the ecosystem by affecting non target organisms like fishes.

Hence, the aim of the present study is: Toxicology studies of Herbicide Pyrazosulfuron-Ethyl on Freshwater Fish (Oreochromis mossambicus): A Sub-Acute study. Thus, to fulfil the aim, following are the objectives:

1. To check the acute toxicity and sub-acute toxicity of Pyrazosulfuron Ethyl to *O. mossambicus.* **(Chapter 1)**
2. To evaluate the Hematological and Biochemical Alterations in *O. mossambicus* exposed to Pyrazosulfuron Ethyl. **(Chapter 2)**
3. To elucidate the altrartion in the oraganosomatic indices of *O. mossambicus* to understand the overall health status. **(Chapter 3)**
4. To determine a histological base health assessment of o. Mossabicus on exposure of Herbicide PE. (**Chapter 4)**
5. To elucidate the genotoxic potential of Pyrazosulfuron Ethyl in *O.mossambicus.*

    4.1 In-vivo genotoxic testing by micronucleus assay.

    4.2To check the cell death using Annexin V-PI Staining. **(Chapter 5)**