CHAPTER 1

INTRODUCTION AND REVIEW
Introduction

Pesticides are being increasingly used for enhancing yield and quality in agricultural produce. The impact of pesticides on non-target organisms depends on their toxicity, metabolites and transport pathways through the hydrological cycle, soil and food chains (Jänsch et al., 2006). Indiscriminate use without following proper guidelines causes the contamination of soil and water and apart from being an operational hazard, posing a serious threat to human health. The risk of acute exposure to these compounds is a constant threat and found to be responsible for numerous cases of poisoning annually in nontarget wildlife and for acute mammalian toxicity and neurotoxicity (Gilden et al., 2010). European Economic Communities (EEC) directive, 1980 fixed the maximum residue levels (MRLs) for drinking water of each individual pesticide at 0.1 μg/l and the total amount of pesticides at 0.5 μg/l.

In order to comply with the strict EEC norms, pesticide residue analysis are performed using chromatographic techniques such as gas chromatography (GC), liquid chromatography (LC) and supercritical fluid chromatography (SFC), together with specific detection, in combination with mass spectrometry (MS) (Schröder, 1993; Hoff and Zoonen, 1999). In general, the above procedures are expensive, time consuming, labour intensive (Krämer, 1996) and also required some toxic solvants (Torres et al., 1996). To avoid the general drawbacks of the above methods, application of immunoanalytical techniques has increased significantly (Suri et al., 2002; Singh et al., 2004). Immunoanalytical techniques are characterized by the high affinity and specificities of the antibody-antigen interaction, offering detection limits at the low parts per billion (ppb) to parts per trillion (ppt) levels. Consequently, these techniques are rapid, simple and cost effective. In addition these can be used by untrained personnel and performed on-site monitoring without requiring sample transfer to an analytical laboratory.

Pesticides are small molecules therefore do not elicite immune response. These molecules are first derivatized (called hapten) containing an appropriate group for attachment to a carrier protein. The size of the spacer arm bridging the protein and hapten in very important for good antigen presentation and 4-6 atoms are often quoted as an optimal range (Szurdoki et al., 1995). Different labels used are radioactive tracers (Fatori and Hunter, 1980), enzymes (Matuszczyk et al., 1996) and fluorescent probes (Niessner et al., 1995).

Recently, antibody technology has evolved as a major tool to detect pesticides whereby monoclonal antibodies with desired specificity can be selected (Kohler and
Milestein, 1975). Antibody genes can be cloned and expressed in various host systems, thus further improving the sensitivity and the reproducibility of immunoassay (Nord et al, 1997; Hoogenboom and Chames, 2000). Exploitation of antibody enzyme conjugate play an important role in recombination technology where the scFvs can be conjugated to other moieties like enzyme by gene fusion technique (Yang et al. 2004; Dhillon et al. 2003) and a luminescent enzyme aquorin have been employed as labels (Casadei et al. 1990). Fluorobodies/luminobodies, the fusion protein product of antibody fragment and fluorescent protein can be produced in culture of bacterial cells (Griep et al., 1999; Schwalbach et al., 2000). Unlike FITC/ RITC/ HRP conjugated antibodies, scFv fusion proteins do not show intensity degradation and have a longer life span. The utilization of fusion proteins is likely to improve detection signal and reduce the time of analysis. Green Fluorescent Protein (GFP) from Aequorea Victoria i.e., which emits green light when illuminated with long wave UV-light showed the promise of being used as a very convenient portable marker for gene expression.

1.1 Pesticides

A pesticide may be a chemical substance, biological agent (such as a virus, fungus or bacteria), antimicrobial, disinfectant or device used against pests including insects, plant pathogens, weeds, mollusks, birds, mammals, fish, nematodes (roundworms) and microbes that compete with humans for food, destroy property, spread or are a vector for disease or are a nuisance.

1.1.1 History

The first known pesticide was elemental sulfur dusting used in Sumeria about 4,500 years ago. By the 15th century, toxic chemicals such as arsenic, mercury and lead were being applied to crops to kill pests. In the 17th century, nicotine sulfate was extracted from tobacco leaves for use as an insecticide. In 1939, Paul Müller discovered that DDT was a very effective insecticide. It quickly became the most widely-used pesticide in the world. However, in the 1960s, it was discovered that DDT was preventing many fish-eating birds from reproducing which was a huge threat to biodiversity. DDT is now banned in most parts of the globe, but it is still used in some developing nations to prevent malaria and other tropical diseases by killing mosquitoes and other disease-carrying insects.

1.1.2 Pesticides: Types and Classification

Today pesticides have become indispensable tools in the efficient control of animals, insects, plants and fungi which otherwise have a detrimental effect on the crop production. There are over 800 pesticides and 20,000 pesticide products, which are currently in use.
Apart from being classified according to the chemical class the pesticide belongs to, they can also be classified broadly into different groups (Sanborn et al., 2002) as

**Insecticides:** are usually organophosphorous (e.g. malathion, chlorpyriphos etc), organochlorine (e.g. DDT) or carbamates (e.g. carbofuran, carbaryl etc). Other insecticide chemicals include Phthalates and hydrazines, Pyrezeoles and Pyrethroids etc.

**Herbicides:** Organic compounds such as Ureas and sulfonylureas, Chlorophenoxy acids, Triazines, Carbamates, benzoic acid, dinitrophenols and Naphthalene acetic acid derivatives (e.g. atrazine, 2,4-D, metachlor etc.) or inorganic compounds (e.g. Arsenicals, Sodium Chlorate).

**Fungicides:** Sulphur and copper salts (copper sulfate etc) or organic compounds such as Azoles, Benzimidazoles, Carboxyimides, Dithiocarbamates and substituted Benzenes (e.g. Captan, Mancozeb etc.)

**Antimicrobials:** Pesticides commonly used as sterilizers, disinfectants, sanitizers, antiseptics and germicides intended to kill or inhibit growth of microorganisms such as bacteria (Bactericides/bacteriostat) include organic chemicals such as Phenols and chlorinated phenols, Hyadantoins, Isothiazolones and Quarternary ammonium salts.

**Rodenticides:** Used to control rodents and related species. Organic chemicals such as Coumarins and Indandiones are used as rodenticides world over.

According to chemical group they can be classified as

**Organophosphate Pesticides:** These pesticides affect the nervous system by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. Most organophosphates are insecticides. They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932.

**Carbamate Pesticides:** Affect the nervous system by disrupting an enzyme that regulates acetylcholine, a neurotransmitter. The enzyme effects are usually reversible. There are several subgroups within the carbamates.

**Organochlorine Insecticides:** Commonly used in the past, but many have been removed from the market due to their health and environmental effects and their persistence (e.g. DDT and chlordane).

**Pyrethroid Pesticides:** were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system.
1.1.3 Pesticides Production and use: Global and Indian Scenario

1.1.3.1 Global Scenario:

In many countries population growth has raced ahead of food production in recent years (United Nations Development Programme, 1997). The world grain harvest increased about 1 per cent annually between 1990 and 1997, less than the average population growth rate of 1.6 per cent in the developing world. Between 1985 and 1995 food production lagged behind population growth in 64 of 105 developing countries studied by FAO. The average amount of grain land per person dropped by almost half between 1950 and 1996 from 0.23 hectares to 0.12 hectares. By 2030, when world population is projected to be at least 8 billion, there would be just 0.08 hectares of grain land per person (Leach and Fairhead, 2000). As for developing countries, in 1992, there were about 0.2 hectares of arable land per person. By 2050, this figure could fall to about 0.1 hectare per capita (Rosenzweig, 2000).

Agriculture is the main source of income for more than 2.5 billion people and 96% of the farmers are living in developing countries. This would require a 40-45% increase in food production. To meet the demand of increase in food production, pesticides are a major farm input with many commercial cash crop operations spending 5-10% of cash operating expenses on these products. The world pesticide market is valued at about $ U.S. 37 billion and India accounts for about 2.5 % of this amount. India is normally ranked 12th in the world pesticide market while the Europe is ranked 1st with close to 33% followed by US with nearly 20% of the global market share. World Pesticide consumption is about 2.5 million tones and Europe is the largest consumer with over 32% followed by the US with around 20%, Asia accounts for about 12% pesticide usage and Canada and Africa are the lowest consumers with about 4% share each, the rest of the world makes for the remaining 20% pesticide consumption (Pimentel, 1995).

The world pesticide, agrochemicals and agriculture trade industry is dominated by a relatively small number of corporations; manufacturers (approximately 15) supplying a large number of active ingredients. According to UN food and Agriculture organization studies (http://apps.fao.org/faostat/default.jsp) it is estimated that 10 of these companies produce 90% of the world's active ingredients (Table 1.1). Just four companies, based in the US and linked in two alliances (Cargill/Monsanto and Novartis/ADM) control over 80% of the world seed market and 75% of the world agrochemical market.
Table 1.1: Major Pesticide Producers of the world along with their Flagship products

<table>
<thead>
<tr>
<th>Serial Number</th>
<th>Company</th>
<th>Prime Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AgrEvo</td>
<td>Liberty</td>
</tr>
<tr>
<td>2.</td>
<td>Cyanamid</td>
<td>Pursuit</td>
</tr>
<tr>
<td>3.</td>
<td>Monsanto</td>
<td>RoundUp</td>
</tr>
<tr>
<td>4.</td>
<td>Dow Elanco</td>
<td>2,4-D</td>
</tr>
<tr>
<td>5.</td>
<td>Novartis</td>
<td>Dual</td>
</tr>
<tr>
<td>6.</td>
<td>BASF</td>
<td>Banvel</td>
</tr>
<tr>
<td>7.</td>
<td>Zeneca</td>
<td>Achieve</td>
</tr>
<tr>
<td>8.</td>
<td>Bayer, Ontario</td>
<td>Furadan</td>
</tr>
<tr>
<td>9.</td>
<td>Rhone-Poulencc</td>
<td>Select</td>
</tr>
<tr>
<td>10.</td>
<td>Bayer</td>
<td>Admire</td>
</tr>
</tbody>
</table>

1.1.3.2 Indian Scenario

Agriculture is the lynchpin of the Indian economy. Ensuring food security for more than 1 bn Indian population with diminishing cultivable land resource is a herculean task. This necessitates use of high yielding variety of seeds, balance use of fertilisers, judicious use of quality pesticides along with education to farmers and the use of modern farming techniques. Use of pesticides in India began in the year 1948 when DDT was imported for Malaria control and the agricultural use started in 1949 with introduction of Benzene hexachloride (BHC) for Locust control (Gupta PK, 2004). The production of pesticides started in the year 1952 with production of BHC followed by DDT production plants. Pesticide Industry in India is second largest in Asia (behind China) and twelved largest in World. In value terms, the size of the Indian pesticide industry was estimated at Rs.74 bn for 2007, including exports of Rs.29 bn. Group wise consumption of pesticides during 1995-2005 in India is show in table 1.2. Among the states, Punjab uses the highest amount of pesticide followed by Haryana (Fig. 1.1). Comparison of pesticide use in India and the world is shown in fig. 1.2.
Table 1.2: Groupwise consumption of pesticides (MT) during 1995-2005 in India

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Insecticide</td>
<td>28,926</td>
<td>26,756</td>
<td>29,839</td>
<td>28,197</td>
<td>25,627</td>
<td>25,929</td>
</tr>
<tr>
<td>Fungicide</td>
<td>8,435</td>
<td>8,307</td>
<td>9,222</td>
<td>10,712</td>
<td>9,087</td>
<td>6,397.4</td>
</tr>
<tr>
<td>Weedicide</td>
<td>7,369</td>
<td>7,299</td>
<td>6,979</td>
<td>7,857</td>
<td>5,610</td>
<td>7,364</td>
</tr>
<tr>
<td>Others</td>
<td>1,465</td>
<td>1,222</td>
<td>1,308</td>
<td>1,398</td>
<td>438</td>
<td>1,660</td>
</tr>
<tr>
<td>Total</td>
<td>46,195</td>
<td>43,584</td>
<td>47,348</td>
<td>48,146</td>
<td>40,762</td>
<td>41,350.4</td>
</tr>
</tbody>
</table>

Figure 1.1: State wise pesticide consumption in India.
1.1.4 Pesticide Pollution and Public Health

1.1.4.1 Pesticide Pollution: A Global Concern

Pesticide use has gained popularity due to their ability to control the pest problem at a very reasonable cost thereby increasing food supplies at lower prices and also in preventing transmission of diseases such as malaria through insects. Pesticides, depending upon their water solubility can either remain in the soil and are broken down by action of microorganisms, or washed off, eventually into surface and ground waters (Rehana et al. 1996; Agarwal, 1999; Suri et al. 2009). The persistency of pesticides and their degradation products in the geosphere causes environmental problems. The transfer of pesticides from treated soil to surface and ground water leads to contamination of drinking water resources and to subsequent intake of pesticides by human populations. They may also present hazards to human health directly as in spray drift of pesticides or indirectly as accumulated pesticide in edible plant or animal tissue.

Rachel Carson was the first to predict a massive destruction of planet’s fragile ecosystem through her book ‘Silent Spring’ (1962). The title of the book was based on the fact that birds died more in the area where pesticides were being used through aerial spraying. She was the first to point out the damage being done to non-target species by uncontrolled use of pesticides through direct and indirect toxicity. Working on chlorinated pesticide she showed that DDT was harmful to fish and crabs and not only to the insects for which it was being used. Indirect toxicity on the other hand was due to the persistency of the pesticides.
**Bioaccumulation**, is the tendency of the persistent pesticides to accumulate and get concentrated in an organism's tissue and **Biomagnification**, is the increase in the concentration of pesticides higher up in the food chain, which leads to indirect toxicity. Carson's work disclosed the fact that even when the pesticide DDT had a very low concentration in water 3 ppt the concentration in zooplanktons was 0.04 ppm, was found to be 0.5 ppm in minnows and further increased to 2 ppm in fish and the birds feeding on fish were found to have a concentration of 25 ppm. In most countries DDT was banned in 1972 but it can still be found in various ecosystems even 3 decades after the ban on its use in seals, fishes, invertebrates, amphibians, birds and human (Ramakant, 2004; Muir et al., 2003; Minh et al., 2002; Wong et al., 2002). It is now evident that even in small amounts these pesticides can cause death, irritate eyes and skin, damage nervous system, disrupt hormonal balance and immune system, effect ability to reproduce and can cause cancer (Hooghe et al., 2000; Garry et al., 1996). Insecticides and herbicides are the two main classes of pesticides, which are most commonly used and are found in environment as pollutants. Due to a large number of chemicals which are being used and deposited in the environment, WHO recommended the classification of pesticides based on the threat it poses to human race, calculated on the basis of Oral and Dermal LD<sub>50</sub> values. Table 1.3 shows the classification categories in which harmful chemicals are divided according to the WHO recommendations.

**Insecticides** are the major cause of concern because the effect produced by their mode of action such as enzyme inhibition, hormonal imbalance etc on the pest animals and insects is also applicable on the humans (Jintana et al., 2009). Acute and long-term exposure to organophosphates leads to elevated neurological, psychiatric symptoms and poor neuropsychological response (Jokanović and Kosanović, 2010). Organochlorine pesticides were used extensively till 1970's but the health hazards associated with their use implied severe restrictions on their use. Organochlorines are known to disrupt hormonal balance, retard growth in children and also adversely affect the immune system (Vine et al., 2000; Karamus et al., 2002).

**Herbicides** owe their indiscriminate use to their effectiveness in controlling weeds and also to their apparent tolerability by animal species including farm animals and humans. Recent studies on long-term exposure to different types of organic herbicides in uses such as heterocyclic aromatic compounds (e.g. Triazines etc.) or phenoxy acids (e.g. 2,4-D etc.) have been reported to cause great concern for human health (Kniewald et al., 2000; Cox, 2001; Narotsky et al., 2001). Phenoxy and triazine herbicides are also known to disrupt hormonal balances (Hayes TB, 2006). Herbicides are linked to increased incidence of cancer and
chromosomal breakage in various studies (Van-Leeuwen, 1999). In an in vitro study triazines were found to change the production of two chemicals, Dopamine and Norepinephrine, both of which act as neurotransmitters in the central nervous system and are thus potential neurotoxic agents (Das et al. 2000, 2001).

Table 1.3: WHO recommended classification of pesticides by hazard

<table>
<thead>
<tr>
<th>Class</th>
<th>Hazard level</th>
<th>LD₅₀ for the rat, oral (mg kg⁻¹ body mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Solids</td>
</tr>
<tr>
<td>Ia</td>
<td>Extremely hazardous</td>
<td>≤5</td>
</tr>
<tr>
<td>Ib</td>
<td>Highly hazardous</td>
<td>5-50</td>
</tr>
<tr>
<td>II</td>
<td>Moderately hazardous</td>
<td>50-500</td>
</tr>
<tr>
<td>III</td>
<td>Slightly hazardous</td>
<td>&gt;500</td>
</tr>
<tr>
<td>III+</td>
<td>Unlikely to present hazard in normal use</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

1.1.4.2 Pesticide Pollution: Indian perspective

Despite the fact that the consumption of pesticides in India is very low, about 0.5 kg/ha of pesticides against 6.60 and 12.0 kg/ha in Korea and Japan, respectively, there has been a widespread contamination of food commodities with pesticide residues, basically due to non-judicious use of pesticides. In India, 51% of food commodities are contaminated with pesticide residues and out of these, 20% have pesticide residues above the maximum residue level values on a worldwide basis. It has been observed that their long-term, low-dose exposure are increasingly linked to human health effects such as immune-suppression, hormone disruption, diminished intelligence, reproductive abnormalities, and cancer (Gupta, 2004).

The first report of poisoning due to pesticides in India came from Kerala in 1958 where, over 100 people died after consuming wheat flour contaminated with parathion (Karunakaran, 1958). Since then several cases of mass poisoning have been reported but minor case still go unreported. Pesticides have been found in food stuffs, human blood, milk and fat (Bhatnagar, 2001). A study on 356 workers in four different manufacturing units of Hexachlorohexane (HCH) showed enhanced neurological symptoms (21%) along with significant increase in detoxifying enzyme levels related to the degree of exposure (Nigam et al., 1993). Rajendran and co workers (1999) reported the presence of Chlorinated pollutants
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in air from a tropical coastal environment (Parangipettai - southeast coast of India). DDT and HCH ranged in concentrations from \(0.16\) to \(5.93\) ng m\(^{-3}\) and \(1.45\) to \(35.6\) ng m\(^{-3}\) respectively. The ban on DDT in agriculture is reflected from the low residue levels recorded, predominantly by metabolites other than the parent compounds. Excessive use of agrochemicals has definitive environmental consequences that have reached alarming levels in Panjab and Haryana, which are also the highest user of agrochemicals per hectare in India (Singh, 2000).

The world's worst industrial disaster occurred in India on the night of December 2-3, 1984, at the Union Carbide plant in Bhopal (population: 900,000) a pesticide manufacturing company. More than 2,500 people died almost instantly, and over 16,000 people have died as a result of health problems related to their exposures to MIC an intermediate product in the manufacture of carbaryl (carbamate pesticide). More than 50,000 people are still suffering significant long term health impacts such as ocular, respiratory, reproductive, immunological, genetic, and psychological (Dhara et al, 2002).

1.1.5 Methods for Pesticide Detection

The conventional methods for detection and analysis of pesticide residues include physico-chemical techniques like Gas Chromatography (GC), High-pressure liquid chromatography (HPLC), Capillary electrophoresis (CE), Mass spectrometry (MS) and their combinations thereof such as GC-MS, LC-MS, GC-MS-MS etc (Osselton and Snelling, 1986).

1.1.5.1 Chromatographic Techniques

a) Thin Layer Chromatography

TLC and HPTLC complement gas chromatography (GC) play a very important role in the determination of pesticide residues, their metabolites and transformation products in environmental waters. They have the following unique advantages over column chromatography: single use of the layer simplifies sample preparation procedures; simplicity of development by dipping the plate into a mobile phase in a chamber; high sample throughput with low operating cost because multiple samples can be run simultaneously with standards on a single plate using a very low volume of solvent. Thin layer radiochromatography (TLRC) is used routinely for metabolism, degradation, and other studies of pesticides in plants, animals, and the environment, and these applications will be covered as well as studies of lipophilicity and pesticide migrations through soils. Screening of
265 pesticides in water by TLC with automated multiple development was published (Hamada and Wintersteiger, 2002).

b) Gas Chromatography:

Gas Chromatography (GC) is most commonly used for separation of thermostable pesticides. Various kinds of fused-silica capillary columns with bonded phases of different polarities are commercially available. The popularity of GC is based on a favorable combination of very high selectivity and resolution, good accuracy and precision, wide dynamic concentration range and high sensitivity (Santos and Galceran, 2003).

Combination of GC with detection methods such as flame ionization detection (FID) and nitrogen–phosphorus detection (NPD) are most popular; FID gives universal response to organic compounds, while NPD is selective for compounds containing nitrogen or phosphorus and gives much lower detection limits than FID. GC–NPD thus is very suitable for detection of amino group containing pesticides. Electron-capture detection (ECD) is used for sensitive determination of the compound containing halogen or nitro group(s) in a molecule (Stalikas and Konidari, 2001).

c) Liquid Chromatography:

Liquid Chromatography (LC) is the method of choice for highly polar, thermolabile and/ or high-molecular mass compounds, which are not amenable to GC. The compatibility of the water samples with the reversed-phase chromatographic separation systems and the possibility of performing derivatization in aqueous solution made LC the preferred technique. Liquid chromatography approach for analysis of pesticides uses various detectors such as ultraviolet (UV), fluorescent Detector (FD) and Refractive index detector (RID) (Kumazawa and Suzuki, 2000). These techniques though highly sensitive, however suffer from several drawbacks

1. Complex sample preparation: Derivatization of the sample to be analyzed is a prerequisite for most of the pesticides, before or after using any particular technique for their efficient detection for example some pesticides such as Glyphosate, bialaphos which contain phosphonic and amino acid groups needs to be derivatized to be less polar and more volatile before they can be detected using GC. The derivatization process may be very lengthy and include the use of highly toxic chemicals (Stalikas and Konidari, 2001). A post chromatographic derivatization to get a fluorophore or chromophore attached to such pesticides for detection has also been reported for detection by HPLC (Oppenhuizen et al., 1991).
2. **Large time for sample analysis**: The chromatographic methods used for the final determination require extraction of the residues from the matrix and subsequent clean-up procedure before they become suitable for analysis (Bruzzoniti *et al.*, 2000). Over 60% of the analysis time is used for sample preparation (Smith, 2002; Krutz *et al.*, 2003; Eskilsson and Bjorklund, 2000).

3. **Sophisticated instrumentation and trained personnel to operate**: Mass selective detection (MS) is now used more often with GC and LC after the mass detectors such as ion trap detectors and bench top quadrupole instruments were improved in their detector design and operation and acquisition software (Kumazawa and Suzuki, 2000). Thus the personnel performing the analysis should be highly trained so as to correctly determine which detector to use for which compound (Stalikas and Konidari, 2001).

4. **Unsuitable for field studies and in situ monitoring of samples**: These techniques due to their bulky and sophisticated instrumentation are limited to the lab and are unsuitable for filed applications. Furthermore, collection, transport and storage can affect sample characteristics, and subsequently, the validity and hence usefulness of analysis. Field analytical methods in combination with an appropriate number of laboratory analyses for field data validation could provide a good monitoring strategy (Mallat *et al.*, 2001; McMohan, 1993; Giese, 2000; Suri *et al.*, 2002).

5. **Large cost of analysis per sample**: The main driving force in the emergence of alternate technology is the increasing cost and number of samples associated with environmental compliance. Therefore, more cost effective tools for water monitoring are urgently needed (Mallat *et al.*, 2001, Nister and Emneus, 1999).

### 1.1.5.2 Electrophoretic Techniques

Recent advances in technology have found application of electrophoresis in the field of environmental pollution monitoring. Small amounts of pollutants can be detected using Capillary electrophoresis, where separation is performed in fused-silica capillaries with internal diameters of 25-100 μm and they provide very high theoretical plate numbers and along with sensitive detection methods such as UV-absorbance and fluorescence make it a highly promising technique. This technique is fast gaining popularity because of its various modes, which can be used for separation of polar as well as non-polar compounds (Martinez *et al.*, 2000).
In capillary zone electrophoresis (CZE) separation is based on differences in the electrophoretic mobility (determined by size and charge) of charged analytes in an electric field. However, since many environmental pollutants are uncharged or have very similar chemical structures, they often cannot be separated from interfering components by CZE and Micellar electrokinetic capillary chromatography (MEKC) has to be used (Altria, 2000).

1.1.5.3 Mass spectrometry

The analysis of organic compounds by mass spectrometry (MS) first involves producing gas phase charged molecular ions and fragment ions of the parent molecules in the ion source of the instrument, and subsequently separating these ions according to their mass-to-charge ratio (m/z), and finally measuring the intensity of each of these ions. A mass spectrometer accomplishes this through a sequence of events within its three main subsystems namely: (i) the ion source in which the ionization of the organic molecules takes place, and from which the ions are then accelerated (or extracted) into (ii) the mass analyzer which separates the ions according to their m/z values, and finally (iii) the detector where the relative intensities (abundances) of the separated ions are determined. The instrument is maintained at low pressure (high vacuum) by a system of turbo molecular or oil diffusion pumps. A mass spectrum displays the values of the m/z ratio for molecular ions and fragment ions along the X-axis, increasing in value from the origin. The relative intensities of the detected ions are displayed on the Y-axis, generally as the % relative abundance compared to the most intense ion in the spectrum. MS has gained popularity more as a detector for specific compounds separated using one of the separation techniques i.e., GC, LC and CE (Careri et al., 1996; Pico et al., 2004).

1.1.5.4 Immunochemical Methods

Immunosensors are based on the binding interactions between immobilized biomolecules (Ab or Ag) on the transducer surface with the analyte of interest (Ag or Ab), resulting in a detectable signal. The sensor system takes advantage of the high selectivity provided by the molecular recognition characteristics of an Ab, which binds reversibly with a specific Ag. In solution phase, Ab molecules interact specifically and reversibly with an Ag to form an immune complex (Ab–Ag) according to the following equilibrium equation:

\[
\frac{Ka}{Kd} = \frac{[Ab-Ag]}{[Ab][Ag]}
\]

where Ka and Kd are the rate constants for association and dissociation, respectively. The equilibrium constant (or the affinity constant) of the reaction is expressed as follows:

\[
K = \frac{Ka}{Kd} = \frac{[Ab-Ag]}{[Ab][Ag]}
\]
The equilibrium kinetics of Ab binding with Ag in solution suggests that both association and dissociation are relatively rapid. The direction of equilibrium depends on the overall affinity, which is basically the summation of both attractive and repulsive non-covalent forces. An immune complex usually shows low Kd values (in the range $10^{-6}$–$10^{-12}$) and also displays higher affinity (i.e. K value typically $10^4$). Immunosensors, which usually incorporate Abs immobilized on solid matrix do not show the same kinetics of reaction established for those reactants in solution. Immobilization of biomolecules on a solid surface can alter the properties of the reactants (measured in reaction-rate constants) or the surface characteristics (e.g., surface charge and hydrophobicity) of the support itself. Until the mid-1980s, immunosensors were mainly used for clinical diagnostics, mostly for the detection of macromolecules (Karube and Suzuki, 1986). Not much work had been reported in environmental monitoring using immunosensors. Development of immunosensors for environmental pollutants, mainly for pesticide analysis was delayed for several reasons. First, getting Abs against pesticide molecules has been a tedious task, primarily because of the low molecular weight of pesticide molecules (usually less than 1 kD), as is evident from the fact that not many Abs against pesticides are available. Second, repeated measurements are less efficient, especially with high affinity Abs (and unlike enzymes). However, development of Ab technologies and advancement in the transducer system have provided further impetus (Kaur et al., 2008; Kim et al., 2003; Singh et al., 2004).

Pesticides, organic compounds of molecular mass, are usually non-immunogenic, so they will not elicit an immune response unless coupled with some macromolecules (e.g., proteins). It is therefore necessary to modify these small substances (haptens) to couple them with macromolecules (carriers) so as to make a stable carrier-hapten complex. The carrier-hapten conjugate developed can be used to generate Ab against pesticides. This section highlights Ab generation and optimization of assay techniques for the detection of pesticide molecules. Abs are specialized proteins capable of recognizing Ags with high specificity. The quality of the Ab employed greatly influences the specificity and the sensitivity of an immunosensor. Polyclonal Abs (pAbs), conveniently prepared from sera, comprise a wide variety of Ab molecules of different specificities and affinity (Hill et al., 1993). Hybridoma technology provides an excellent alternative, whereby monoclonal Abs (mAbs) can be produced in unlimited quantities with constant characteristics. Besides, mAbs of desired affinities can also be selected. Recombinant DNA technology has been exploited to engineer recombinant Abs (rAbs), whereby Ab genes can be cloned and expressed in various host systems, thus further improving the sensitivity and the reproducibility of immunoassay
(Scheller et al., 2001). The choice between pAbs, mAbs or rAbs for development of an immunosensor depends upon the various aspects of the assay (e.g., cost of development, relative specificity and sensitivity of the Abs, ease of screening and selection of Abs) (Suri et al., 2008).

1.1.5.4 Classification of immunosensors

In general, immunosensors can be distinguished from immunoassays where the transducer is not an integral part of the analytical system. If a transduction is achieved using labeled species, the principles are similar to immunoassays. Depending on if labels are used or not, immunosensors are divided into two categories: labeled type and label-free type.

1.1.5.4.a Labeled formats

This procedure involves a label to quantify the amount of Ab or analyte bound during an incubation step. Widely used labels involve enzymes (e.g. glucose oxidase, horseradish peroxidase (HRP), β-galactosidase and Alkaline phosphatase (AP), nanoparticles, and fluorescent or electrochemiluminescent probes. (Wilson et al., 1997; Keay and McNeil, 1998; Danielsson et al., 2001; Seydack, 2005; Wilson, 2005). Fig. 1.3 shows the schematic of labeled immunosensors. Commonly, two different formats for labeled immunosensors are available: sandwich assays and competitive assays. A sandwich assay consists of two recognition steps. In the first step, the Ab is immobilized on a transducer surface, allowing it to capture the analyte of interest. In the second step, labeled secondary Ab is added to bind with the previously captured analyte. The immunocomplexes (immobilized Ab-analyte-labeled Ab) are formed and the signals from labels increase in proportion to the analyte concentration (Sadik and Van Emon, 1996). In competitive assays, the analyte competes with labeled analyte for a limited number of antibody binding sites. As the analyte concentration increases, more labeled analyte are displaced, giving a decrease in signal if antibody-bound labeled analyte is detected (Bange et al., 2005).

Use of fluorescence for detection provides a very sensitive method of detection. If these substances are excited by polarized light, the molecules change their orientation and the emitted light is also depolarized. Fluorescence polarization (FP) is determined by exciting these compounds with vertically polarized light and measuring the intensity of both the vertically (Iv) and horizontally (Ih) polarized components of the fluorescence light emitted. The polarization (P) value is determined as
Figure 1.3: Principle of labeled immunosensor. Concentration of analyte to be monitored is correlated with the amount of labeled antigen (A) or labeled antibody (B) to the corresponding ligand coated on the transducer surface.

The ratio of the difference and the sum of Iv and Ih components:

\[ P = \frac{I_v - I_h}{I_v + I_h} \]

Theoretically, the value of \( P \) is 0 or 0.5 for full depolarization or fixed molecules without movement, respectively. The use of FP-based immunoassay (FPIA) for the detection of pesticides has been described extensively (Eremin, 1998). FPIA measures the increase in FP when a tracer (fluorophore-labeled Ag) is bound to a specific Ab; the FP signal is decreased when free analyte competes with the tracer for binding. FPIA for small pesticide molecules is a competitive method based on detection of the difference of FP between a small fluorescent-labeled Ag and its immune complex with a specific Ab. When there is no analyte present in the sample, the tracer will be completely bound to the specific Ab and the FP value will increase. However, if the analyte concentration in sample is greater than the concentration of the tracer, the Ab-binding sites will be occupied by the analyte so there will be free tracer. This will decrease the FP value of the reaction mixture.

Sensitive method based on an optical immunosensor was developed to determine different types of organic pollutants in water samples (Rodríguez-Mozaz et al., 2005). The system, called river analyzer (RIANA), is based on a rapid, solid-phase, indirect, inhibition immunoassay that takes place at an optical transducer chip chemically modified with an analyte derivative. Fluorescence produced by labeled Abs bound to the transducer is detected.
by photodiodes and can be correlated with analyte concentration. Similarly, a parallel affinity-sensor array (PASA) system, based on chemiluminescence labels (peroxidase/luminol) and charge-coupled device (CCD) detection was developed for monitoring pesticide contaminants in water (Weller et al., 1999). An LOD of this system of 20 ng/l was achieved for terbutylazine. The assay could be regenerated more than 100 times.

After the chemiluminescence (CL) phenomenon of luminal reported by Albrecht in 1928, investigation of effective catalysts for such CL reactions has increasingly been carried out, including metal ions, metal complex, and enzymes. The catalyzed luminal CL has been successfully applied in bioanalysis and immunoassay or as sensitive detectors for high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE).

Recently, gold nanomaterials as biological labels for immunoassays have attracted keen interest for their unique size dependent optical and electronics properties and great analytical potential (Rosi and Mirkin, 2005; Seydack, 2005; Daniel and Astruc, 2004). These nanoparticles have been widely used for their catalysis of gas / liquid phase redox reaction (Wallace and Whetten, 2002) and also as catalyst for some chemiluminescence (CL) reactions (Zhang et al., 2005; Cui et al., 2005) in liquid phase systems. In a CL immunoassay format based on gold nanoparticles, Fan et al., (2005), developed a stripping CL immunoassay which was based on the dissolving of gold nanoparticles to Au (III) after immunoreactions and reacting with luminol to produce CL signals. However, these methods employed stripping procedure, i.e., dissolution of gold nanoparticles under extremely strict conditions, which could result in high CL background. In a non-stripping CL immunoassay based on gold nanoparticles, Li and co-workers (2007) demonstrated that the gold nanoparticles with irregular geometry could greatly enhance the CL intensity of the luminol-H$_2$O$_2$ system.

A luminol-peroxide chemiluminescent system has attracted particular interests in the field of bioanalytical chemistry. For generating the light emission, the oxidation of luminol proceeds for the first step. Metal ions and organometallic compounds are known to catalyze the oxidation of luminol with peroxide in strong alkaline solution (pH 10-13) (Huang et al., 1996; Obata et al., 1993). Table 1.4 shows some examples of immunosensors using labels for the determination of residual pesticides.
Table 1.4: Some examples of immunosensors using labels for the determination of residual pesticides

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Detector</th>
<th>Label</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
<td>Fluorescent</td>
<td>Nano-particle</td>
<td>ELISA</td>
</tr>
<tr>
<td>Chlorsulfuron</td>
<td>Amperometric</td>
<td>Glucose-oxidase</td>
<td>0.01-1 ng/ml</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>Fluorescent</td>
<td>Glyphosate-peroxidase</td>
<td>0.021 ng/ml</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>Fluorescent</td>
<td>Fluorescein</td>
<td>0.1 ng/ml</td>
</tr>
<tr>
<td>Simazine</td>
<td>Potentiometric</td>
<td>HRP</td>
<td>3 ng/ml</td>
</tr>
<tr>
<td>Mesotrione</td>
<td>Fluorescent</td>
<td>Fluorescein</td>
<td>0.04 ng/ml</td>
</tr>
</tbody>
</table>

1.1.5.4.b Label-free formats

This procedure detects the binding of pesticide and the Ab on a transducer surface without any labels. There are also two basic types in this format: direct and indirect. In the first type, the response is directly proportional to the amount of pesticides present. The vital advantage of these direct immunosensors is the simple, single-stage reagentless operation. However, such direct immunosensors are often inadequate to generate a highly sensitive signal resulting from Ab–Ag binding interactions and it is still difficult to meet the demand of sensitive detection. The second type, also based on competitive formats, is carried out as a binding inhibition test. The antigen (pesticide–protein conjugate) is first immobilized onto the surface of a transducer, and then pesticide-antibody mixtures are preincubated in solution. After being injected on the sensor surface, the antibody binding to the immobilized conjugate is inhibited by the presence of target pesticide. It is advanced transducer technology that enables the label free detection and quantification of the immune complex (Fig. 1.4). It has been proven to be an effective method for the determination of pesticide. Some examples of label-free immunosensors for the determination of residual pesticides are presented in Table 1.5.
Introduction and Review

Membrane potential changes

Electrode potential changes

Piezoelectric properties change

Optical properties change

Figure 1.4: Principle of labeled free immunosensor. Antigen binding to antibody-coated surface changes the physical properties of the transducer that can be converted into detectable electrical signal.

Table 1.5: Some examples of label-free immunosensors for the determination of residual pesticides

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Pesticide</th>
<th>Detector</th>
<th>Detection limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2,4-D</td>
<td>Impedimetric</td>
<td>45 nmol/l</td>
<td>Navr’atilov’a and Skladal (2004)</td>
</tr>
<tr>
<td>2.</td>
<td>2,4-D</td>
<td>SPR</td>
<td>0.1 ng/ml</td>
<td>Gobi et al. (2007)</td>
</tr>
<tr>
<td>3.</td>
<td>Atrazine</td>
<td>Impedimetric</td>
<td>20 ng/ml</td>
<td>Hleli et al. (2006)</td>
</tr>
<tr>
<td>4.</td>
<td>Atrazine</td>
<td>Impedimetric</td>
<td>8.34±1.37 ng/ml</td>
<td>Valera et al. (2007)</td>
</tr>
<tr>
<td>5.</td>
<td>Atrazine</td>
<td>Piezoelectric</td>
<td>1.5 ng/ml (direct)</td>
<td>P’ribyl et al. (2003)</td>
</tr>
<tr>
<td>6.</td>
<td>Chlorpyrifos</td>
<td>SPR</td>
<td>45–64 ng/l</td>
<td>Mauriz et al. (2006a)</td>
</tr>
<tr>
<td>7.</td>
<td>DDT</td>
<td>Nanomechanical</td>
<td>Below nM range</td>
<td>Alvarez et al. (2003)</td>
</tr>
<tr>
<td>8.</td>
<td>Trifluralin</td>
<td>Optical waveguide</td>
<td>100 ng/ml (direct)</td>
<td>Sz’ek’acs et al. (2003)</td>
</tr>
</tbody>
</table>

i) Piezoelectric crystal immunosensor

The PZ crystal immunosensor works on the principle of measuring a small change in resonant frequency of an oscillating PZ crystal due to a change in mass on the sensor surface, as a result of the formation of the Ab–Ag complex. The resonant frequency of a PZ crystal is mass dependent, giving LODs for analyte concentration at the sub-ng level (Suri, 2006). Fig. 1.5 (A–C) shows the PZ crystal vibrating in thickness shear mode and the formation of immunocomplex (Ab–Ag) on electrodes. Fig. 1.5 D shows a prototype of a quartz-crystal
microbalance (QCM) system that our group developed with an auto-flow attachment (patent filed) for pesticides monitoring. The basis of this mass-frequency dependency [i.e. the surface mass change (Dm) and resonant frequency shift (DF) of a PZ crystal vibrating at fundamental frequency (F)] is attributed to the Sauerbrey equation (Sauerbrey, 1959):

$$\Delta F = -k F^2 \frac{\Delta m}{A} = -2.26 \times 10^{-6} F^2 \Delta m/A$$

where $\Delta F$ is the change of frequency (Hz) due to coating, $k$ is the proportional constant depending upon density and shear modulus of quartz crystal (for AT-cut quartz, the density is 2.648 g/cm$^3$ and shear modulus is 2.947 X $10^{11}$ dynes/cm$^2$), $F$ is the fundamental frequency (MHz) of the quartz crystal, $\Delta m$ is mass (g) of coating deposited, and $A$ is the coated area of the crystal (cm$^2$). The oscillating frequency of the quartz crystal immersed in a aqueous medium is also influenced by the density ($\rho$) and the viscosity ($\eta$) of the medium, as described by the following equation (Kanazawa and Gordon, 1985):

$$\Delta F = -2.26 \times 10^{-6} F^2 \left( \frac{\rho}{\eta} \right)^{1/2}$$

This equation gives a shift in fundamental frequency of the order of 7 kHz for a 10-MHz quartz crystal with only one face in contact with aqueous medium. Characterization of mAbs to 2,4-D using a PZ crystal microbalance in solution has also been reported. Because of their low cost and high quality factors (Q), these miniaturized sensors have fast response time and high sensitivity, and they can be mass-produced using standard fabrication techniques. (Steegborn and Skla’dal, 1997)

![Figure 1.5](image)

**Figure 1.5:** (A) Piezoelectric crystal construction, (B) crystal vibrating in thickness shear mode, (C) piezoelectric crystal bound with receptor molecules for antigen detection, and (D) quartz-crystal set-up that our group designed and developed for pesticide analysis. The quartz-crystal microbalance (QCM) unit is coupled to a flow-through system for on-line monitoring applications.
ii) Electrochemical immunoensors

 Formation of Ab–Ag complex in electrochemical transducers alters the change in ion concentration or electron density on the electrode surface, which, in turn, is measured by electrodes. Electrochemical transducers, classified as amperometric, potentiometric, conductimetric and capacitative, measure changes in current, potential (voltage), conductance and capacitance, respectively. Applications of electrochemical transducers for detection of different pesticide molecules have been reported. A rapid assay based on an immunoenzyme electrode and peroxide conjugates was developed for 2,4-D and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) (Dzantiev et al., 1996). The assay monitors the competitive binding of free pesticide and pesticide-peroxide conjugate with anti-pesticide Ab immobilized on a graphite electrode by measuring peroxide activity in the immune complex on the electrode surface. LODs for 2,4-D and 2,4,5-T were about 50 ng/ml showing no interference with serum protein present in solution. A simple electrochemical immunoSENSOR-based assay for the field-based quantification of 2,4-D in soil extracts has been demonstrated (Kroger et al., 1998). The sensor utilized a competitive immunoassay format, incorporating an immobilized Ag complex at the surface of a disposable, screen-printed, working electrode element. The extent of glucose oxidase-labeled Ab binding to the Ag electrode was determined amperometrically and was related to the sample-analyte concentration. A disposable immunoSENSOR with liposome enhancement and amperometric detection technique for monitoring herbicide triazine in real samples has also been presented (Baumner and Schmid, 1998).

 In a different approach, an acetylcholine (Ach) receptor-based electrochemical biosensor was developed (Eldefrawi et al., 1998). The Ach receptor was fixed to the gate of an ion selective field-effect transistor (ISFET) and binding of Ach with the receptor resulted in a potential change that was detected by the ISFET.

 iii) Micromechanical immunoSENSORs

 The merging of silicon-microfabrication techniques with surface-functionalization chemistry offers an exciting new opportunity in developing ultra-sensitive microscopic immunoanalysis devices. Micromechanical transducers make use of cantilevers as force sensors in an atomic force microscope (AFM), which can transduce a signal caused by the formation of immune complex onto the cantilever surface into a mechanical motion with high sensitivity (Browning-Kelley et al., 1997). The origin of this nanomechanical bending of such a hybrid structure has been shown to be by a change in the surface stress due to ligand–receptor binding (Wu et al., 2001), so detection of nanomechanical bending offers a quantitative measure of both the occurrence and the kinetics of specific molecular binding.
events on the microcantilever beam. The selectivity of molecular interactions is dictated by the molecular probe (receptor) layer on the microcantilever surface, while the sensitivity is governed by the extent of microcantilever deflection resulting from the molecular binding event (Raiteri et al., 2000; Thaysen et al., 2000). Binding of 2,4-D to its mAb was monitored on a thin gold layer (30 nm) deposited on a cantilever. Binding characteristics of anti-2,4-D Ab to the protein-2,4-D conjugate were analyzed by measuring the bend of cantilever due to Ab–Ag interaction on its surface. Recently, we demonstrated label-free detection of highly-specific atrazine Ab–Ag interactions at the nm scale on microcantilevers up to ppt sensitivity (Suri et al., 2008).

Fig. 1.6 shows the preparation of the thiolated anti atrazine Ab and the corresponding site-directed immobilization scheme on the gold-coated cantilever. The absolute deflection of the cantilever, Dz, was measured using an optical detection system in liquid with constant flow of Ag (Sentric System, Veeco Instruments). The deflection signal resulted in a differential surface stress, Δσ, so, using Stoney’s equation:

$$\Delta \sigma = \frac{1}{4} \left( \frac{t}{L} \right)^2 E/(1-\nu) \Delta z;$$

where L is the effective length of the cantilever, t is the thickness, and E/(1-\nu) is the ratio between the Young’s modulus, E (130 GPa), and Poisson ratio \( \nu \) of Si (0.28). Because the cantilever deflection also strongly depended on the geometry, all the cantilevers used in these measurements were exactly similar in their geometry. As a result, the surface-stress change due to chemical interactions was directly related to quantitative measurements for the bindings.
iv) Surface-plasmon resonance

In a different evanescent format, using SPR, the excitation is provided by the evanescent field, which is produced at the site of total internal reflection from a glass-metal interface. In this format, laser light first enters the optical substrate at certain angle and then strikes the glass-metal boundary by first passing through the layer coated with biomolecules on the metal film before contacting the glass. The intensity of the reflected light is a function of the refractive index at the glass-metal interface. Any change in the refractive index, caused by the formation of the immune complex on the metal surface, signifies immunological recognition (Fig. 1.7). The SPR phenomenon was first utilized in an immunosensing
application by Liedberg and his team (Chegel et al., 1998). Immunosensor systems based on SPR detection have been developed for various pesticides in aqueous solutions (Liedberg et al., 1998; Mouvet et al., 1997). An inhibition immunoassay using the BIAcore system achieved an LOD of 0.005 ppb for atrazine in water with the analysis time of approximately 15 min. A competitive immunoassay based on SPR for detection of pesticide 2,4-D was reported recently (Svitel et al., 2000). The interaction between anti-2,4-D Ab and the surface-bound concanavalin A-2,4-D conjugate was monitored by SPR and the response was used to quantify 2,4-D. The dynamic range of the curve was 3–100 ng/ml. SPR-based immunoassays for small molecules have been reviewed by Mullett and his colleagues (Mullet et al., 2000).

v) Evanescent wave

An optical immunosensor based on EW combines the potential of molecular recognition by the Ab with the good signal-transduction capability of a waveguide or fiber-optic probes. Signal generation can be obtained directly or enhanced by coupling a reactant with a label. When light is propagated through a waveguide (g1) by multiple total internal reflections in the x direction, an electromagnetic (EM) wave (called an EW) is generated in the optically less dense external medium (g2) with g1 > g2. For visible light, the evanescent field penetrates 50–500 nm in the y direction, so only surface-bound molecules interact with the incident light. This allows the absorption of energy by the molecules located in the evanescent field, leading to attenuation of the light reflected in the waveguide. For immunosensing applications, planar-waveguide systems exploit the same optical phenomenon as fiber optics with some minor variations (Marazuela, 2002). The waveguide can be mass-produced at very low cost by injection molding. A suitable detector monitors the formation of Ab–Ag complex. A major advantage of this system is minimal interference from the bulk media. There are two possible methods for measuring EW: attenuated total reflection (ATR) and total internal reflection fluorescence (TIRF). In ATR mode, the energy absorbed by the surface bound molecules is monitored as an attenuation of the internally reflected light. Since the absorbed light is usually a small percentage of the total incident light, the sensitivity of the assay is very poor. In TIRF mode, the evanescent photons captured by surface-bound molecules are re-emitted at a longer wavelength as fluorescence. Fig. 1.7A shows the basic principle of an EW biosensor where light is directed into the waveguide and generates an EM field, allowing excitation of fluorophores used as labels with Abs or Ags. EW biosensors gave limits of detection (LODs) for 2,4-D and simazine were 0.035 ng/ml and 0.026 ng/ml, respectively (Klotz et al., 1982).
Figure 1.7. Evanescent wave (EW)-based optical immunosensor. (A) Optical waveguide, and (B) surface-plasmon resonance (SPR) configuration. The monochromatic laser light is directed into the prism/waveguide, which generates an electromagnetic (EM) field allowing excitation of fluorophores used as tracers in optical waveguide and resonance shift in SPR, respectively.

1.2 Hapten Synthesis

Hapten, a derivative of the target molecule, contains an appropriate group for attachment to carrier protein. Direct coupling of haptens with carrier proteins is possible if the target compound contains functional groups (e.g., −NH₂, −COOH, −CHO, or −SH). However, it is necessary to derivatize the target compounds such as pesticide with such functionality before conjugation with carrier protein. Design of haptens for linking with carrier proteins is the most important aspect of specific Ab generation against pesticide molecules. Hapten molecules are synthesized so that they:

- Mimic the structure of the compound; and,
- Contain a reactive group that can form a covalent linkage with the carrier proteins.

Minimal structure alteration to the hapten ensures that the Ab recognizes the unmodified hapten in the assay system. The hapten should also preserve to a great extent the chemical structure and the spatial conformation of the target compound. Careful hapten selection might be important for generating class-specific or compound-specific Abs for the immunosensor system. Class specificity of the Abs developed could be achieved by preserving Ag determinants common among related compounds. Similarly, masking these common determinants and targeting a distinct molecular entity could obtain compound specificity (Kim et al., 2003).
For the bioconjugation of hapten with carrier-protein molecules, the functional group of the hapten governs the selection of the conjugation method to be employed. However, the stability of the hapten while conjugating with the carrier protein is an important factor. The problem of hapten stability during conjugation has been highlighted by various groups (Hill et al., 1993; Harrison et al., 1991). It is also important that the hapten-protein conjugate is designed so as to preserve and enhance the epitopic regions on the hapten for getting an Ab specific against a target molecule. The site of linkage of hapten and protein should usually occur away from any suspected Ag determinants. Using a spacer arm (bridging groups) between hapten and protein structure also helps in the production of hapten-specific Abs. The spacer arm can be envisioned as a pedestal for the hapten to accommodate recognition and binding by the large Ab molecule. In general, increasing the length or the hydrophobicity of the spacer arm helps generating Abs of higher specificity and good titer. The same hapten employing a shorter spacer arm may not elicit the Ab response. Design of a suitable hapten and selection of a spacer arm and a suitable carrier protein are therefore critical to ensure successful generation of Abs having the desired specificity (Kim et al., 2003).

Immunogens contain a B-cell epitope and a T-cell epitope, both of which are necessary for the production of antibodies against a particular antigen of interest. When hapten-labeled proteins are introduced into a suitable host animal, they generate antibodies against hapten or protein or hapten protein conjugate (Brinkley, 1992). In addition to these intrinsic groups, specific reactive groups may be introduced into the protein by chemical modification. Some of the reactive groups found on proteins are amines, thiols, phenols, and carboxylic acids.

1.2.1 Bioconjugate Preparation

1.2.1.1 Protein Modification Reagents

There are different reagents that are available for the purpose of protein modification. These reagents are designed to have specific reactivity with functional groups contained in each reactant (Annunziato et al., 1993) and may be grouped according to the reactive groups that they target on the protein to be modified (Brinkley, 1992). These may be grouped as follows:

a) Amine- Reactive Reagents are those that react primarily with lysines and α-amino groups of proteins to give amide products. \textit{N}-hydroxy-succinimide (NHS) ester of S-acetylthioacetic acid (SATA) is one of the commercially available amine-reactive reagents. These reagents have intermediate reactivity toward amines, with a high selectivity toward aliphatic amines.
Other reagents that have been used to modify amines of proteins are acid anhydrides (Brinkley, 1992).

b) Thiol-Reactive Reagents are those that will couple to thiol groups on proteins to give thioether-coupled products (Brinkley, 1992). Maleimides react with thiols resulting in formation of a thioether bond.

c) Carboxylic Acid-Reactive Reagents. Amines can be coupled to carboxylic acids of proteins via activation of the carboxyl group by a water-soluble carbodiimide followed by reaction with the amine (Brinkley, 1992) and resulting in formation of stable amide bonds.

d) Bi-functional reagents or cross-linking reagents are specialized reagents that will form a bond between two different groups, either on the same molecule or two different molecules (Brinkley, 1992). Those with the same reactive group at each end of the molecule are called homobifunctional while those with different reactive groups at each end of the molecule are called heterobifunctional. Succinimidyl (acetylthio) acetate (SATA) contains both an amine reactive and a protected thiol group. After de-protection, the thio-containing protein is reacted with a thiol-reactive group on the other protein. p-Maleimidophenyl isocyanate (PMPI) is another heterobifunctional cross-linking agent containing a thiol reactive (maleimide) and a hydroxyl reactive group (Annunziato et al., 1993). The isocyanate group reacts with amines to form ureas and with alcohols to form carbamates. The heterobifunctional reagents allow coupling to be carried out in a stepwise manner and result in better control of the conjugation chemistry (Annunziato et al., 1993).

1.2.1.2 Carrier Proteins

A carrier protein is a large molecule capable of stimulating its own immune response (Harlow and Lane, 1988) and it must come from a species different from the animal to be immunized. The carrier molecule provides the T-cell epitope for the production of a successful immune response (De Silva et al., 1999). The proteins to be used as carriers must possess functional groups that can easily be substituted (Brinkley, 1992). Many different carrier proteins have been used for coupling with peptides to create immunogens. The choice of the carrier to use is based on immunogenicity, solubility, and whether adequate conjugation with the hapten can be achieved. Keyhole limpet hemocyanin (KLH) is one of the commonly used carriers, however due to its large molecular weight (~ 8,000,000 Da) the serum antibody response to the carrier often obscures that of the antigen of interest (De Silva et al., 1999) resulting in a reduced antigen-specific antibody concentration in the IgG fraction.
1.3 Antibody generation and characterization

Antibodies have become important research tools for many applications including the detection and purification of desired targets. Antibodies are host proteins produced in response to the presence of foreign molecules in the body (Harlow and Lane, 1988). They are synthesized primarily by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage, and circulate throughout the blood and lymph where they bind to foreign antigens. An antibody response however is the culmination of a series of interactions between macrophages, T lymphocytes and B-lymphocytes all reacting to the presence of a foreign material (antigen). The extreme specificity at the molecular level of each immunoglobulin for its antigen has made antibodies valuable therapeutic and diagnostic tools. Monoclonal and polyclonal antibodies are useful in a variety of circumstances for their different and unique properties.

1.3.1 Polyclonal Antibodies

A general protocol of immunization, for the generation of either pAbs or mAbs, involves injecting the immunogen mixed with the adjuvant (first using complete Freund’s adjuvant followed by boosters with Freund’s incomplete adjuvant) at multiple sites. Sera are then collected for the isolation of pAbs. For mAb generation, spleen cells of an immunized animal are fused with an appropriate myeloma cell line, and extensive screening is carried out to select a clone secreting the mAb desired. In our earlier work (Kaur et al., 2008), we extensively described production and characterization of Abs against low molecular-weight pesticides (e.g., atrazine) with major emphasis on immunization protocols. Cross-reactivity of an Ab to unexpected metabolites is a major problem in any immunodetection technique for pesticide applications. Depending upon the conjugate used for immunization and the class of chemicals under investigation, cross-reactivity of the Abs with metabolites similar to the analyte of interest are frequently observed (Harrison et al., 1991). Cross-reactivity, in general, could be minimized by using compound-specific (monospecific) pAbs or mAbs, because there are then fewer binding epitope-recognizing analogues and metabolites other than the target compound.

The principal animals that have been used as polyclonal antibody sources are rabbits, sheep, goat and donkey; however, there has been a surge of interest in the utilization of chicken egg-yolk immunoglobulin in recent years (Losso et al., 1998). Chicken antibody, or 7S immunoglobulin, is referred to as chicken IgG or IgY (Losso et al., 1997). The antibody in egg yolk differs in molecular weight and isoelectric point from mammalian IgG (Hansen et
IgY originates from the serum and is transferred preferentially by the follicular epithelium of the ovary to the developing ova via specific receptors 4-5 days before ovulation; it ranges from 9 to 25 mg/ml of egg yolk (Losso et al., 1997). IgY is transported from the hen to the egg to protect the offspring against infections until the chick’s immune system has matured so that it can provide the young animal with sufficient amounts of antibodies. The active transport of IgY from the serum to the egg results in a higher concentration in the yolk than in the serum and therefore more antibodies can be produced per month in a laying hen than in a rabbit (Larsson and Sjöquist, 1990). Immunoglobulins (IgY) from egg yolk can be a valuable source of antibodies when obtained from hens immunized against the target antigen. The advantages that IgY offers over conventional sources of antibody include (1) the potential to produce gram quantities (2) low cost of production, the cost of feeding and handling is considerably lower for a hen than for a rabbit, (3) purification of IgY is relatively simple, and (4) the convenience of collecting antibodies from eggs rather than bleeding animals, which facilitates compliance with animal welfare considerations (Losso et al., 1998 and Losso et al., 1997). Chicken antibodies also have biochemical advantages over mammalian antibodies (e.g., rabbit antibodies) that can be used to improve immunoassays where antibodies are used. Due to great evolutionary distance between birds and mammals, a chicken is able to produce antibodies against more epitopes on a human antigen than a rabbit, which will give a stronger signal in immunological assays (Carlander et al., 2003). Antibodies are purified from the yolk usually by separating the lipid fraction from the water-soluble faction. Several methods have been used for the purification of IgY. Polson et al., (1985) used polyethylene glycol precipitation, Jensenius et al., (1981) used dextran sulfate precipitation, Hassl and Aspöck (1988) used hydrophobic interaction chromatography and gel filtration to obtain pure egg yolk immunoglobulins. Hatta et al., (1990) used xanthan gum precipitation. Akita and Nakai (1992) used a water dilution method in which water-soluble plasma proteins were separated from the granular proteins as the egg yolk granules aggregated with dilution. Factors that affect this purification method are extent of dilution, incubation time, pH of diluted egg yolk and addition of sodium chloride. This method was employed in this study. Akita and Nakai (1993) compared the water dilution method with the polyethyleneglycol, dextran sulfate and xanthan gum methods in terms of yield, purity, ease of use, potential scaling up and immunoactivity of IgY. They reported that the water dilution method gave the highest yield, followed by dextran sulfate, xanthan gum and polyethylene glycol method in that order. The underlying principle for the purification of
IgY by the four methods compared is the precipitation of granules leaving the IgY in the water-soluble fraction or supernatant.

1.3.2 Monoclonal Antibodies

Presence of an immunogenic molecule in an animal stimulates B-lymphocytes (B cells), which undergo proliferation, differentiation, and maturation such that numerous B cells produce antibodies to combat the invasion. Each B-cell produces a single type of antibody molecule (monoclonal) such that the overall response involves different antibody molecules (polyclonal) from different B-cells (Liddell and Cryer, 1991). Monoclonal antibody production aims at selecting a B-cell producing a single type of antibody that is specific to the antigen and proliferating it in vitro to produce the desired amount of antibody. The cells that are isolated from the immunized animal, however, do not continue to grow in tissue culture on their own. Kohler and Milstein (1975) showed that the property of permanent proliferation could be added to the B-lymphocyte by fusing it with a tumorigenic plasma cell (myeloma) from the same animal species. The myeloma cells provide the correct genes for continued cell division and the antibody secreting cells provide the functional antibody genes. The hybrid cell or hybridoma can then be maintained in vitro and will continue to secrete antibodies with a defined specificity. Myeloma can be induced in a few strains of mice by injecting mineral oil into the peritoneum (Harlow and Lane 1988). Potter (1972) isolated myelomas from BALB/C mice and they are the most commonly used partners for fusion. Polyethylene glycol (PEG) is the most common fusogen for hybridoma production. It fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei (Harlow and Lane, 1988). Usually, myeloma has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis (Liddell and Cryer, 1991). Selection with 8-azaguanine yields a cell line harboring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (HGPRT). The addition of any compounds (azaserine, aminopterin, etc) that block the de novo nucleotide synthesis pathway will force the cells to use the salvage pathway. Cells containing nonfunctional HGPRT protein will die in these conditions, while the hybrids between myelomas with a nonfunctional HGPRT and antibody secreting cells (B-cells) with a functional HGPRT will be able to grow.

1.3.3 Phage Display Library

Animal immunisation followed by hybridoma technology has been used to generate monoclonal antibodies against a variety of antigens. Over the past ten years, advances in molecular biology have allowed for the use of *Escherichia coli* to produce recombinant
antibodies. By restricting the size to either a Fab, a Fv or a linker-stabilised single chain Fv (scFv) such antibody fragments cannot only be expressed in bacterial cells but also displayed by fusion to phage coat proteins (Griffiths and Duncan, 1998). The phage display concept was first introduced for short peptide fragments in 1985 (Smith, 1985). Fragments of the EcoRI endonuclease, displayed as a polypeptide fusion (phenotype) to the gene 3 protein (g3p), were encoded on the DNA molecule (genotype) encapsulated within the phage particle. The linkage of genotype to phenotype is the fundamental aspect of phage display. Subsequently, phage display of functional antibody fragments was shown (McCafferty et al., 1990) when the VH and VL fragments of the anti-lysozyme antibody were introduced, with a linker, into a phage vector at the N terminus of g3p. Since then, large scFv, Fab and peptide repertoires have been generated using a variety of phage display formats. One of the major advantages of phage display technology of antibody fragments compared with standard hybridoma technology is that the generation of specific scFv/Fab fragments to a particular antigen can be performed within a couple of weeks (Arndt et al., 2001).

1.4 Problem Definition: Overall Aims and Objectives

This study was aimed at the development and characterization of a highly specific, sensitive, reliable and cost effective method for detection and monitoring of pesticides in environmental samples. Biosensors have of late become a method of choice for environmental monitoring. Consisting of a biological detection element in close association with a physical transducer gives a fast method of monitoring biomolecular interactions. The specific interaction of biomolecules with target analyte generates a physical response and the transducer detects this physical change and produces a corresponding electronic signal. We chose antibodies for use as biological recognition element because the vertebrate immune system is practically capable of producing antibodies specific for any target analyte provided it is able to initiate an immune response. Thus the overall aim of the current study was to develop and characterize immunobiosensors for detection of pesticides in environmental samples.

1.4.1 2,4-Dichlorophenoxyacetic acid

2,4-D has a molecular weight of 221.0 and a molecular formula of C₈H₆O₃Cl₂. It is soluble in organic solvents. The reported solubility of the free acid in water varies considerably and is given as 0.09 % or 900 mg/l at 25°C. The phenoxy herbicides, and 2,4-D in particular, ushered in the chemical weed control revolution in the mid 1940's. Even 51 years after its introduction, 2,4-D continues to be the most commonly and widely used
herbicide worldwide. Monochloroacetic acid and 2,4-dichlorophenol were used to make 2,4-D. Subsequently, salts were made by adding the appropriate amine or inorganic hydroxide to the acid. Esters were synthesized by reacting 2,4-D with the appropriate alcohols. A systemic pesticide moves inside a plant following absorption by the plant. This movement is usually upward (through the xylem) and outward. Increased efficiency may be a result. Systemic insecticides which poison pollen and nectar in the flowers may kill needed pollinators.

**Physical Properties:**

<table>
<thead>
<tr>
<th>Table 1.6: Chemical characteristics of 2,4-D</th>
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<tbody>
<tr>
<td>Molecular weight</td>
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<tr>
<td>Melting point</td>
</tr>
<tr>
<td>Boiling point (at .4 mmHg)</td>
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<tr>
<td>Water solubility (average of two values near 25°C at pH7)</td>
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<tr>
<td>Vapor pressure(at 25°C)</td>
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<tr>
<td>Hydrolysis half-life (average of two values at 25°C, pH7)</td>
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<tr>
<td>Aqueous photolysis half-life (at 25°C)</td>
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<td>Anaerobic aquatic half-life</td>
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<td>Aqueous aerobic half-life</td>
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<td>Aerobic half-life</td>
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<tr>
<td>Soil photolysis half-life</td>
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<td>Field dissipation half-life</td>
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1.4.2 Discovery and development of 2,4-D

Botanists have long been intrigued with plant shoot and root growth and the mechanisms causing plants to respond to stimuli. Kögl and Haagen-Smit, 1934 reported the isolation of indole acetic acid (IAA) from plants and human urine and identified it as the principal naturally occurring hormone (later called an auxin) in plants. Because IAA was unstable outside of plants, researchers began synthesizing and investigating the effect of IAA derivatives and homologs on plant growth activity. Pokorny in 1940 synthesized 2,4-D and 2,4,5-T while looking in vain for a fungicide. In June 1941, Pokorny described the chemical synthesis of 2,4-D.

Kraus at the University of Chicago had observed since 1936 that certain growth regulators were phytotoxic (Peterson, 1967) and in 1941 he was first to propose that growth
regulators might work as herbicides, because they often killed test plants. In November 1942, the United States Army began developing Camp Detrick in Frederick, Maryland, as the center for research and testing of chemicals for biological warfare with special emphasis on crop destroying chemicals.

In November 21, 1962 United States and South Vietnam military personnel sprayed 2,4,5-T plus 2,4-D (Agent Orange) in Vietnam to defoliate trees along military routes to reduce sniper activity. All biocidal research activities were terminated in 1972, following a declaration by President R. M. Nixon that the United States would no longer conduct biological warfare research or develop biological weapons.

In 1942, Zimmerman and Hitchcock reported that the phenoxy acetic acids could induce seedless tomatoes and that they were potent synthetic plant hormones. Franklin D. Jones with the American Chemical Paint Company (ACPC) filed on March 20, 1944 and received use patent 2,390,941 in December 1945 for 2,4-D as a herbicide. In June 1945, ACPC marketed 2,4-D under the brand name Weed one, which was the first selective, systemic herbicide produced and sold on a commercial scale.

1.4.3 Mode of action

2,4-D is thought to increase cell-wall plasticity, biosynthesis of proteins and the production of ethylene. The abnormal increase in these processes is thought to result in uncontrolled cell division and growth, which damages vascular tissue. 2,4-D is generally applied to the foliage of broadleaf plants or directly to the soil as both a liquid and a granular product. It is also applied as an aqueous solution to sapwood cuts in the bark known as a hack and squirt application. Plants absorb 2,4-D through their roots and leaves within 4-6 hours after application (Munro et al., 1992). Following foliar absorption, 2,4-D progresses through the plant in the phloem, most likely moving with the food material. If absorbed by the roots, 2,4-D moves upward in the transpiration stream (EPA, 1988). Accumulation of the herbicide occurs in the meristematic regions of the shoots and roots. 2,4-D mimics the effect of auxins, or other plant growth regulating hormones, and thus stimulates growth, rejuvenates old cells, and over stimulates young cells leading to abnormal growth patterns and death in some plants (Mullison, 1987). Plants treated with 2,4-D often exhibit malformed leaves, stems, and roots. 2,4-D affects plant metabolism by stimulating nucleic and protein synthesis, which affects the activity of enzymes, respiration, and cell division (EPA, 1988). Often, cells in the phloem of treated plants are crushed or plugged, interfering with normal food transport (Mullison, 1987), which can leave parts of the plant malnourished or possibly lead to death.
Introduction and Review

1.4.3 Impacts of 2, 4-Dichlorophenoxyacetic acid

In mammals, 2,4-D disrupts energy production (Zychlinksi and Zolnierowicz, 1990), depleting the body of its primary energy molecule, ATP (adenosine triphosphate) (Palmiera et al., 1994). 2,4-D has been shown to cause cellular mutations, which can lead to cancer. This mutagen contains dioxins, a group of chemicals known to be hazardous to human health and to the environment (Littorin, 1994). Numerous epidemiological studies have linked 2,4-D to non-Hodgkin’s lymphoma (NHL) among farmers (Zahm, 1997; Fontana et al., 1998, Zahm and Blair, 1992, Morrison et al., 1992). Multi-center studies in Canada and in Sweden of members of the general public found 30-50% higher odds of 2,4-D exposure among people with NHL (McDuffie et al., 2001, Hardell and Eriksson, 1999, Sterling and Arundel, 1986). The teratogenic, neurotoxic, immunosuppressive, cytotoxic and hepatoxic effects of 2,4-D have been well documented (Blakley et al., 1989; Sulik et al., 1998; Barnekow et al., 2000; Rosso et al., 2000; Venkov et al., 2000; Charles et al., 2001; Madrigal-Bujadar et al., 2001; Osaki et al., 2001; Tuschl and Schwab, 2003). Other researchers publishing in the open scientific literature have reported oxidant effects of 2,4-D, indicating the potential for cytotoxicity or genotoxicity. For example, Bukowska (2003) reported that treatment of human erythrocytes in vitro with 2,4-D at 250 and 500 ppm resulted in decreased levels of reduced glutathione, decreased activity of superoxide dismutase, and increased levels of glutathione peroxidase. These significant changes in antioxidant enzyme activities and evidence of oxidative stress indicate that 2,4-D should be taken seriously as a cytotoxic and potentially genotoxic agent. 2,4-D causes significant suppression of thyroid hormone levels (Rawlings et al., 1998). Similar findings have been reported in rodents, with suppression of thyroid hormone levels, increases in thyroid gland weight, and decreases in weight of the ovaries and testes (Charles et al., 1996). The increases in thyroid gland weight are consistent with the suppression of thyroid hormones, since the gland generally hypertrophies in an attempt to compensate for insufficient circulating levels of thyroid hormones. Thyroid hormone is known to play a critical role in the development of the brain. Slight thyroid suppression has been shown to adversely affect neurological development in the fetus, resulting in lasting effects on child learning and behavior (Haddow et al., 1999). 2,4-D causes slight decrease in testosterone release and significant increase in estrogen release from testicular cells (Liu et al., 1996). In rodents, this chemical also increases levels of the hormones progesterone and prolactin, and causes abnormalities in the estrus cycle (Duffard et al., 1995). In Minnesota, higher rates of birth defects have been observed in areas of the state with the highest use of 2,4-D and other herbicides of the same class. This increase in birth
defects was most pronounced among infants who were conceived in the spring, the time of
largest herbicide use (Garry et al., 1996). 2,4-D also interferes with the neurotransmitters
serotonin and dopamine. Females are more severely affected than males. Rodent studies have
revealed a region-specific neurotoxic effect on the basal ganglia of the brain, resulting in an
array of effects on critical neurotransmitters and adverse effects on behavior (Bortolozzi et
al., 2001; Rosso et al., 2000). This herbicide specifically appears to impair normal deposition
of myelin in the developing brain (Duffard et al., 1996). The neurotoxic and anti thyroid
effects of 2,4-D make it highly likely that fetuses, infants, and children will be more
susceptible to longterm adverse health effects from exposure to this chemical although they
may appear normal at birth. Young animals can also be exposed to 2,4-D through maternal
milk (Sturtz et al., 2000).

2,4-D is a moderately persistent chemical with a half-life between 20 and 200 days.
Unfortunately, the herbicide does not affect target weeds alone. It can cause low growth rates,
reproductive problems, changes in appearance or behavior, or death in non-target species.
Due to the widespread use of 2,4-D on agricultural land, the environmental effects of this use
are emerging in scientific studies. Donald et al., (1999) found agricultural pesticides in
wetlands, and 2,4-D was the most commonly detected pesticide.

Thus keeping above described observations in mind, our objective is aimed at
developing a sensitive system for detection of 2, 4-D in soil and water. The brief outlines of
the objectives are

OBJECTIVES
1. Hapten synthesis, bioconjugate preparation and characterization.
2. Antibody generation in mice/rabbit/chicken their characterization and affinity
   purification.
3. Labeling of antibody with fluorophore/nanoparticles for immunoassay development.
4. Development of immunochemical methods for biomonitoring of 2, 4-D in water and soil.
5. Optimization and validation of developed immunochemical technique.
1.5 Bibliography


