Validation study of Paper-based biosensor for detecting pesticides in real world samples

Kanchana Mysore Somashekar

mysorek@mcmaster.ca

VALIDATION STUDY OF PAPER-BASED BIOSENSOR FOR DETECTING PESTICIDES IN REAL WORLD SAMPLES

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Kanchana Mysore Somashekar

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AUTHOR: Kanchana Mysore Somashekar

SUPERVISORS: Dr. Carlos Filipe and Dr. Robert H. Pelton

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Abstract

Research in paper-based analytical devices has been increasing in recent years. Before technology transfer and market acceptance, these paper-based sensors have to be validated with field samples. In this study, we have made an attempt to evaluate the effectiveness of paper-based sensors to detect pesticides in real world samples. Generation 1 biosensor has been modified to be user friendly. There is no difference in the performance of generation 2 sensors; they detect pesticides based on colorimetric assay. The assay protocol involves first introducing the sample to the sensing zone by pipetting the water sample. Following an incubation period of 15min, the substrate end of the sensor is dipped into the sample to move paper bound indoxyl acetate (IDA) to the sensing region to initiate the enzyme-catalyzed hydrolysis of the substrate, resulting in the development of blue color. The presence of pesticide is indicated by either a decrease in color intensity or with no color development at all.

To evaluate the effectiveness of biosensor in detecting pesticides in real world samples, a field study was conducted in four villages of southern India. Water samples from different aquatic environment including both surface water and ground water, were tested using generation 2 paper-based sensors. The paper-based sensors were capable of detecting organophosphorus pesticides in real world samples. The results were confirmed using GC-MS.

The presence of higher concentration of dibutyl phthalate (in the range of 100uM to 10mM) in water can be a potential interference for the paper-based assay for the detection of pesticides in water. The paper-based biosensor assay platform can detect pesticides in the environmental samples and results have been validated by GC-MS. But for transfer of technology to the industry, further optimization is required to improve the stability of substrate to withstand atmospheric temperature fluctuations thus allowing the storage and shipment of

the biosensor strips. Additionally to conduct reliable assays and obtain consistent results, the fabrication of biosensor strips needs to be improved to maintain the consistent volumes of bioinks impregnated on paper support.

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Table of Contents

List of Figures

List of Tables

Acronyms and abbreviations used in the text

AChE	Acetylcholinesterase enzyme
DT_{50}	Half-life in soil
EU	European union
FPD	Flame photometric detector
GUS	Groundwater ubiquity score
GC	Gas chromatography
IDA	Indoxyl acetate
K_{oc}	Soil-sorption constant
K_{ow}	Octanol / water partition coefficient
LC	Liquid chromatography
MRL	Maximum residual limit
MS	Mass spectrometry
NPD	Nitrogen phosphorus detector
OPPs	Organophosphorus pesticides
TPs	Transformation products
UV	Ultraviolet

1. INTRODUCTION

Biosensor:

The National Research Council (part of the U.S. National Academy of Sciences) has defined a biosensor as, “a detection device that incorporates a) a living organism or product derived from living systems (e.g., an enzyme or antibody) and b) a transducer to provide an indication, signal or other form of recognition of the presence of a specific substance in the environment”. A biosensor is self-contained assimilated receptor-transducer device and consists of a biological recognition element in intimate contact or assimilated with a transducer [1]. Biosensors have been envisioned to play a significant role as a rapid device for detecting analytes of interest in the field of medicine, industrial, agricultural and environmental monitoring.

1.1 Paper based biosensors:

VTT (a Finnish research organization) defined bioactive paper as “paper–like products, cardboard, fabrics and their combinations, etc., with active recognition and/ or functional material capabilities” [2]. Paper has been widely used in everyday life for packaging, writing and as hygiene products, from the time of its invention. Paper filters have extensive applications in analytical chemistry for filtration purpose and chromatography support. In recent years, significant research initiatives have been taken in developing bioactive-paper sensors for detecting different analytes in the field of medicine, biochemical and environmental analysis. The advantages of using paper as support for developing diagnostic devices are:

1) Paper is manufactured from recyclable and renewable resources. It is cheap and is manufactured locally in most part of the world.
2) Paper is biodegradable and can be easily burned.
3) Paper can be easily altered by coating, printing and impregnation. It can be easily stacked, transported.

4) The pores structure of the paper enables it to work as a filter and facilitates lateral-flow assays and chromatographic separations.
5) Paper is made of cellulose or cellulose-polymer mixture and is compatible with proteins and biomolecule friendly, thus facilitates biological applications.
6) The surface chemistry and porosity of paper affects the wetting properties, which is essential for bioactive paper production.

One of the major research initiatives in this area is the development of paper-based biosensors for the detection of different analytes. Incidents of contaminated food and water and pollutants in the environment and the consequent risks involving human health has raised the need for the development of a rapid, reliable, portable and low-cost device. These devices will be particularly beneficial to remote places and less industrialized parts of the world. These devices can detect different analytes in water and food, and will be more affordable compared to sophisticated laboratories which need skilled personnel [2].

The history of paper-based sensors dates back to 1956, when Marion H. Cook and team reported the development of paper-based sensor for the detection of glucose in urine. The sensor was fabricated by impregnating glucose oxidase peroxidase and orthotolidine. It is an enzyme based assay and the presence of glucose in urine is signaled by appearance of blue color. The development of this simple, specific and sensitive test indicates potential usefulness in urinalyses in the medical field for diabetes detection screening [3].

Recently, a team from Harvard University lead by Whitesides developed paper-based diagnostic device for urine analysis. A pattern is created on the paper which acts as a channel and controls the flow of biological fluids. These devices measure glucose and protein concentrations in the urine. This paper-

based urine analysis is based on a colorimetric assay. White color of the paper eases the color analysis by providing contrast background [4, 5].

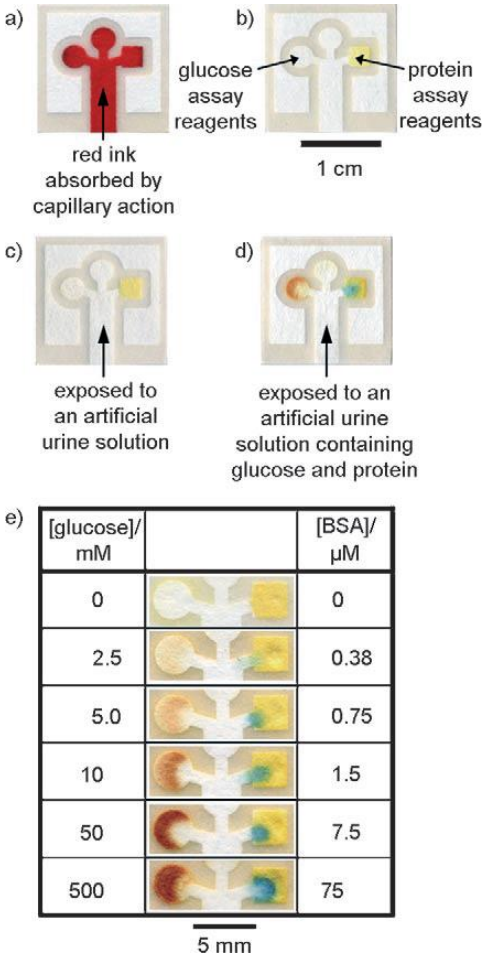


Figure 1.1: an example of urine analysis on a paper-based chromatographic diagnostic device. Artificial urine (5µl) is used to run the assay, color change from clear to brown indicates the presence of glucose. Positive result in case of protein is indicated by color change from yellow to blue [4].

The digital image of the color obtained was further analyzed to obtain the concentration. A following publication from the same group showed quantification of the bioassay by analyzing the color intensity. Digitizing of the color intensity was done using the camera on a mobile phone and sent to

experts' offsite for further quantification of the bioassay [5]. These developments in the paper-based diagnostic tools indicate the potential application of paper-based diagnostic devices in remote places and developing countries.

Another development in paper-based diagnostic devices, with a slightly different approach is paper microzone plates. It was developed as an affordable alternative to plastic microliter plates. Paper microzone plate was fabricated by patterning sheet of paper into a group of circular or square test zones. Because of the natural wicking capability of the paper, solution added to the paper plate will concentrate rapidly by evaporation and thereby enhances the sensitivity of detection. It was demonstrated that these plates can be digitized and quantified using a scanner. This development has provided a strong platform in the area of low cost diagnostic devices. It has the potential to find broader application in remote places and developing countries, in the field of medicine, food, water and the environment [6].

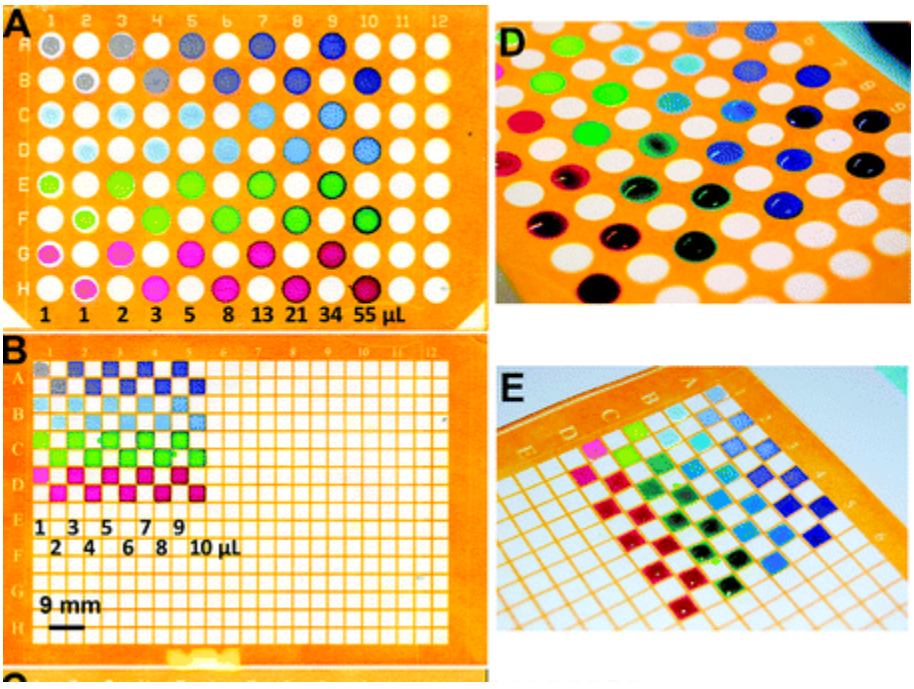


Figure 1.2: Examples of microzone plates, 96 wells (A, D), 384 wells (B, E) [7].

Many studies have been reported by different researchers on paper-based bioassays for detecting pathogens, toxins in food, water and environment. Most of these paper-based sensors are developed by immobilizing biosensors/biomarkers on a paper support. Different types of biosensors based on the target of analysis are used to construct the paper-based sensors, which includes enzyme [8, 9], DNA aptamers [10], phages [11], and cells [12].

One recent paper–based functional dipstick/ lateral-flow biosensor to detect neurotoxins (organophosphate and carbamate pesticides) was developed by Brennan's research team at McMaster University. Immobilization of protein on the paper support is an essential requirement in fabricating paper based biosensors. In this work, the enzyme was immobilized in between two layers of biocompatible sol-gel derived silica on paper. Biosensors were fabricated as follows: Whatman #1 paper was cut in pieces of dimension (1 X 10cm) on which AChE (500U/ml) and IPA (3mM) were impregnated in two regions (sensing and substrate). The sensing region was fabricated by entrapping PVAm/silica/AChE/silica layers in the order described, while substrate region was prepared by entrapping silica/IPA/silica layers using piezoelectric inkjet printer DMP-2800, Fujifilm Dimatix, Inc. (Tokyo, Japan). This paper–based sensor is based on colorimetric assay (Ellman's Assay), and detects acetylcholinesterase inhibitors. Acetylcholinesterase hydrolyses the red-yellow colored IPA, to the blue-purple indophenoxide anion (IDO^-). The absence or decrease in blue-purple color indicates the presence of pesticides. It was demonstrated that use of the cationic polymer, polyvinylamine, to create a capturing region proved to retain the strong colored indophenoxide anion product of the enzyme-substrate reaction. This device was demonstrated to use as (1) directly, normal lateral flow-based assay and as an (2) inverted lateral flow-based device. In case of inverted lateral flow, the sample is flowed directly into the AChE region without exposure to IPA by immersing the top of the strip into the sample. The strip is then inverted and the bottom of the strip is immersed into ddH_2O to move IPA to the sensing area

of the sensor to generate signal. These dipsticks were sensitive enough to detect up to 100nM paraoxon and 30nM aflatoxin B1, these are extremely hazardous compounds because of their potent toxicity to the human nervous system [9, 13].

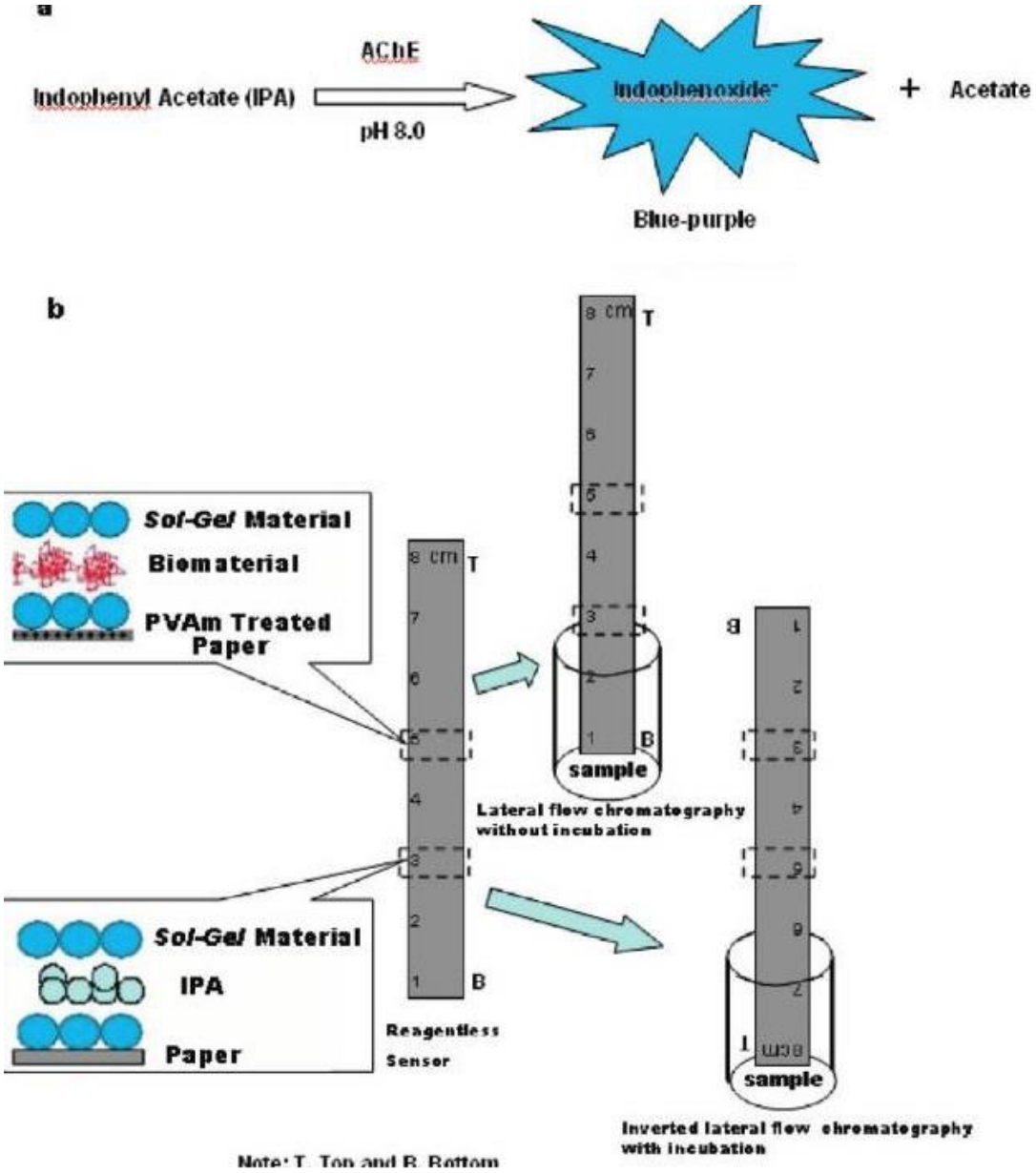


Figure 1.3: (a) Schematic diagram of the detection principle of the indophenyl acetate (IPA)-based colorimetric assay. (b) Biosensor depicting sensing and substrate regions and example of sensor usage in two different ways (1) the sensor can be used directly without incubating the contaminated sample (2) inverted lateral-flow assay with incubation of the sample [13].

In a later publication, this team reported the development of a rapid, selective and a sensitive biosensor to detect heavy metals. The detection of heavy metals is based on the colorimetric measurement of the enzyme activity of β- galactosidase (B-GAL). Both the enzyme and substrate (CPRG) were printed in between two layers of sol-gel derived silica in two regions (sensing and substrate regions) on the paper support. Bioink was printed using an inkjet printer and a layer of hydrophobic barrier (either wax or methyltrimethoxysilane) was printed on top of the sensing zone to avoid the leaching of colored product. These sensors are capable of detecting heavy metals individually and in mixture, in ten minutes time. Presence of heavy metals is indicated by decrease in the color intensity of red-magenta colored product produced by the enzyme substrate reaction. Sensors demonstrated the detection limit for different heavy metals as follows: Hg(II) - 0.001 ppm, Ag(I) - 0.002 ppm, Cu(II) -0.020 ppm, Cd(II) - 0.020 ppm, Pb(II) - 0.140 ppm, Cr(VI) - 0.150 ppm, Ni(II) - 0.230 ppm. This paper-based assay showed immunity towards the interferences from nontoxic ions like Na^+ and K^+. The results from the validation study of biosensor strips, with tap water and lake water were compatible with conventional methods [14].

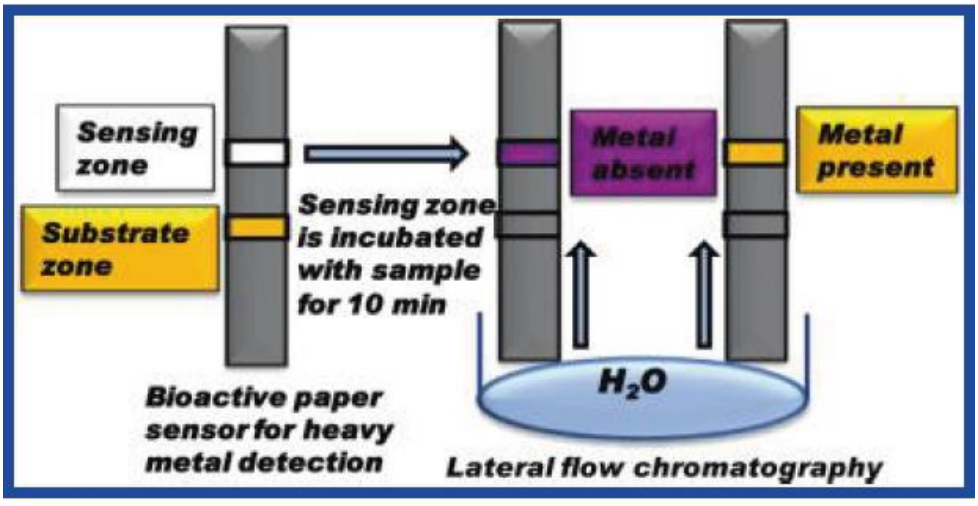


Figure 1.4: Example of lateral flow based biosensors for detecting heavy metals [14].

In one of the most recent publications, Henry's team from Colorado State University, reports development of paper-based analytical device for the detection of foodborne pathogens. This paper- based analytical device detects E-coli, Salmonella Typhimurium and L. monocytogens in food samples. The bioassay is based on chromogenic substrate and species-specific enzyme. µPADs were fabricated by creating a pattern of array of spots on a simple well-plate design. Bioassay was conducted in the paper wells. The presence of pathogens is indicated by change in the color. This paper–based analytical device displayed the sensitivity to detect pathogens upto 10^1 cfu/cm^2 in inoculated ready to eat meat. This device gives quick results within 8-12 h depending on target species, whereas the conventional culture technique requires 5-7 days to detect and identify foodborne pathogens [15]. The development of paper-based, quick, reliable and low-cost µPAD devices gives a hope for application in remote places and the developing world. As this device is less time consuming and affordable compared to traditional method which is time consuming and expensive.

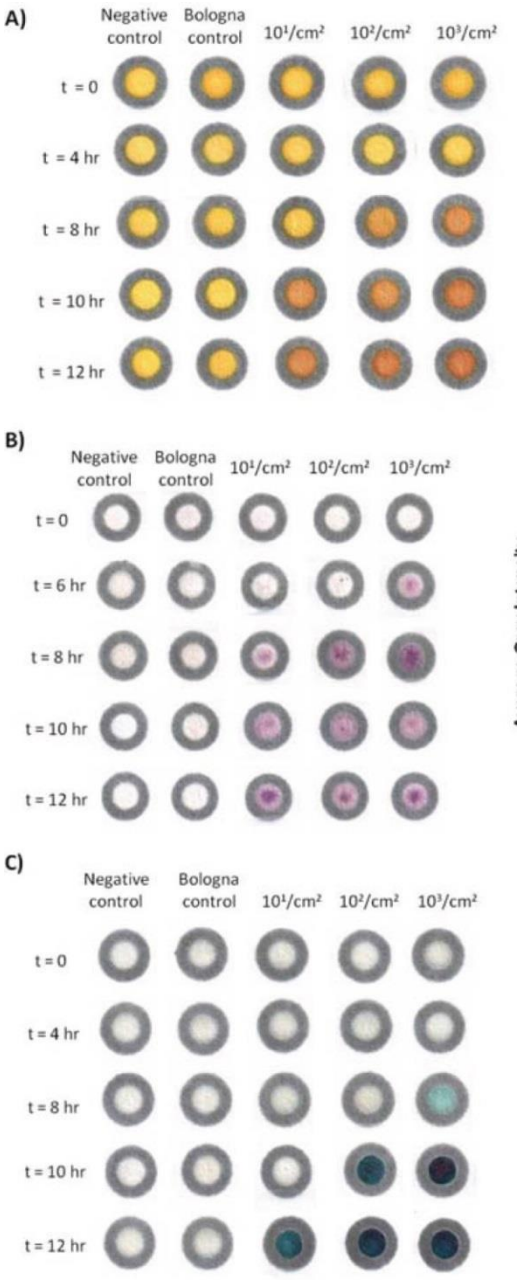


Figure 1.5: Example of analysis of RTE meat samples spiked with different concentrations of (A) E.coli O157:H7, (B) Salmonella Typhimurium and (C) L. Monocytogenes. Samples were tested for enzyme activity with time [15].

1.2 Pesticides:

Pesticides have been extensively used all over the world since the middle of twentieth century. Pesticides are applied to protect plants from disease, insects, pests, and weeds, in the agricultural, domestic and industrial sectors. Pesticides usually come into contact with soil and reaches surface water by runoff and ground water by leaching through the soil. The fate of pesticides in soil depends on the physical, chemical and biological dynamics of the matrix (mobility, sorption, plant uptake, volatilization, run-off, chemical, biological degradation and photodegradation) (Figure.1.6) [16, 17].

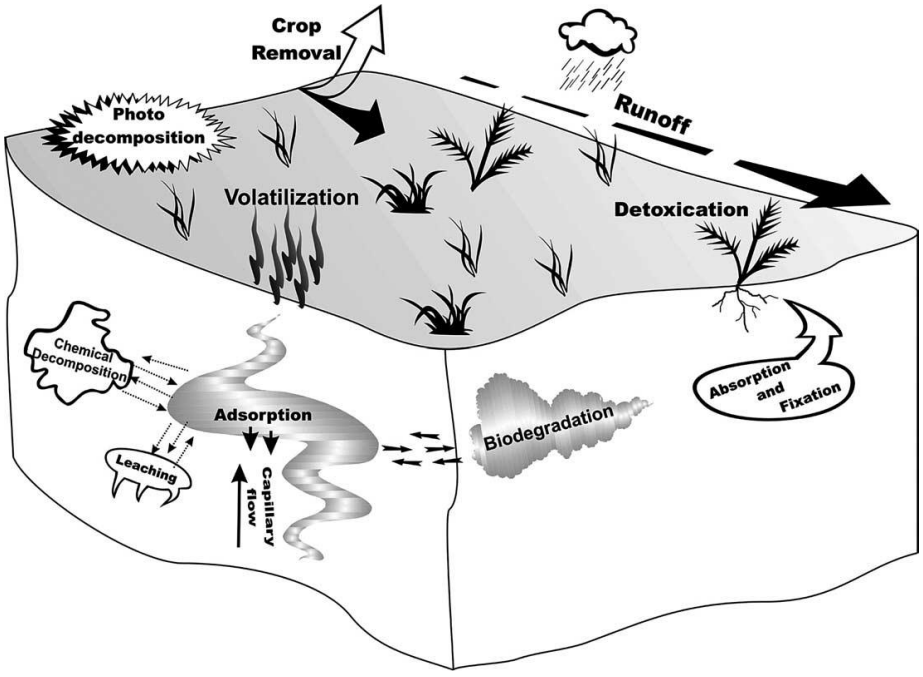


Figure 1.6: Fate of pesticides in soil [16].

The persistence of pesticides defines the “lasting power” in the environment where the chemicals are applied. Most pesticides degrade or break down by chemical and biological process. The chemistry of pesticides, together with the environmental conditions influences the rate of degradation of pesticides. Temperature, water pH, microbial activity and other characteristics of soil may influence pesticide persistence. Chemical degradation includes

reactions like hydrolysis, oxidation and reduction. Biological degradation takes place when microorganisms break down or consume pesticides. Microbial activity usually decreases when soil pH becomes extremely alkaline or acidic, however these conditions may favor rapid chemical degradation. Pesticides may also breakdown by sunlight; this process is termed photodegradation and is influenced by intensity of spectrum of sunlight, length of exposure and properties of pesticides. Some pesticides form “metabolites” or transformation products during degradation, ultimately being completely degraded into inorganic constituents [16-18].

Persistence and movement of the pesticides and their transformation products depends on water solubility, soil-sorption constant (K_{oc}), octanol/water partition coefficient (K_{ow}), and half-life in soil (DT_{50}) [19]. The potential of pesticides to contaminate ground water depends on the “partition coefficient” (PC) of the soil. PC is defined as “the ratio of pesticide concentration in the adsorbed state and the solution phase” [17]. The smaller the value of PC, the amount of pesticides entering the ground water will be high [16, 17]. One of the other parameters which decides the movement of pesticides into ground includes groundwater ubiquity score (GUS). GUS is obtained by persistence of pesticides and binding ability of pesticides to soil particles. It is a numerical value and derived from the half–life and sorption coefficient (GUS= $\log_{10}$(1/2 life) X [4 – $\log_{10}$ (K_{OC})]. The higher the GUS value, the higher the potential for pesticides to move toward groundwater [18].

Table 1.1: Partition coefficients (PC) for selected pesticides [17]

Pesticide	**PC**	**Pesticide**	**PC**
Aldicarb	10	Malathion	1778
Carbofuran	29	Methyl parathion	7079
Fenuron	34	Parathion	7161
Carbaryl	229	Chloropyrifos	13,490
Diuron	389	DDT	243,000

Table 1.2: Persistence of pesticides in soil [17]

Non-Persistent (half-life less than 30days)	**Moderately Persistent (half-life greater than 30days, less than 100)**	**Persistent (half-life greater than 100 days)**
Aldicarb	Aldrin	Bromacil
Malathion	Carbaryl	Chlordane
Capton	Carboduran	Lindane
Dalapon	Parathion	Picloram
Methyl parathion	Phorate	

Pesticides are classified into organochlorines, organophosphorus, carbamates, triazines, thio and dithiocarbamic acids, hetrocyclic N- compounds, urea and phenolic pesticides.

i) Organochlorines: they are group of hydrocarbons with one or more chlorine atoms. Examples: Endosulfan, dieldrin, DDT.
ii) Organophosphates: these are integrated by esters of phosphoric, phosphonic, phorothionic or related acids. Commonly used in this group are Malathion, Parathion, Dimethoate, Paraoxon.

iii) Carbamates: they are compounds formed by salts or esters of carbamic acid. Examples are carbaryl, carbofuron.
iv) Triazines: made up of a number of substituted 1,3,5 – triaines.
v) Ureas: They are mainly herbicides and comprises of large number of groups as phenylureas, sulfonylureas or benzoylureas [20].

Based on the chemical nature, pesticides and their transformation products (TPs) could be grouped into:

i) *Polar pesticides:* majorly herbicides [21, 22]] represent this category but it also includes carbamates [23], fungicides and some organophosphorus insecticide TPs. They are easily moved into surface water by runoff and into ground water by leaching.
ii) Hydrophobic, persistent and bioaccumulable pesticides. They will be strongly bound to soil, organochlorines and their TPs exhibit such behavior [16].

Pesticides are well known contaminants of environment, usage of pesticides is beneficial in agriculture for protecting crops from pests, insects and thus increases the crop production. But, pesticides enter the food-web through bioaccumulation and can eventually become a threat to both humans and animals. Over the past many decades, the usage of organochlorine pesticides is banned because of its long persistence in environment. Organochlorine compounds are replaced by counterparts organophosphates (OPPs) because of their ability to degrade more easily in the environment. Organophosphates may also be used in combination with carbamate pesticides for the control of pests showing persistence to OPPs compounds [24-26].

Because of their wide usage, organophosphorus pesticides are commonly found in water resources and food [24-26]. OPPs inhibit acetyl- cholinesterase and thus cause serious threat if absorbed by humans [27]. The European Union (EU) allows a maximum residual concentration of 0.1µg/L of each individual

pesticide and 0.5 µg/L of the sum of pesticides in drinking water (European Union Drinking Water Directive, 98/83/EC) [27].

1.3 Analysis of organophosphorus and carbamate pesticides:

Several studies have been reported on the analysis of pesticides residues in aqueous samples [25-28]. Determinations of trace amount of organophosphorus pesticides in aqueous samples are carried out using gas chromatography (GC) with nitrogen phosphorus detection (NPD) [24, 29, 30], flame photometric detection (FPD) [25, 27, 31] or mass spectrometry (MS) [29, 32] and liquid chromatography with UV or mass spectrometry detection [33]. But, gas chromatography is not applicable for thermally labile carbamates pesticides. The use of liquid chromatography is suitable for thermo sensitive pesticides.

Prior to analytical determination of the trace pesticides in aqueous samples, low concentrations of analytes in complex matrices must be recovered. Pesticides are usually extracted using liquid-liquid extraction [27], solid-phase extraction [29, 33] and solid phase micro extraction [24, 30, 34]. Liquid-liquid extraction often requires large amount of toxic solvents and the procedure is time consuming. Disposal of large quantities of expensive solvent to the environment is a major concern with this method. Solid phase extraction is less time consuming and is preferred over liquid-liquid extraction. But, solid phase extraction column needs pretreatment and also requires solvent for elution [25].

Solid phase micro extraction is the recent commercially available technique for concentrating low concentrations of OPPs from complex matrices. Solid phase micro extraction device consists of two major components, a syringe and fiber. The syringe serves as holder for the fiber assembly, fused-silica fiber is of small diameter and it is been coated with a liquid polymeric stationary phase. The coated fiber will be directly exposed to sample or head space above the sample, allowing the absorption of the analytes based on their affinity towards

the fiber coating. This method is solvent free, easy and fast which makes it advantageous over the conventional liquid-liquid extraction and solid phase extraction methods. However, it also suffers from drawbacks; its fiber is brittle and has a limited lifespan [24, 25]. Cantwell's group was the first to develop a liquid–liquid microextraction, in which extraction was achieved into a single drop [35]. In the later publications Cantwell and Lee reported the development of the single drop microextraction technique. It was developed as a solvent-minimized sample extraction technique, which uses a small volume of solvents and minimal exposure to toxic solvents [36].

The table below shows the advantages and disadvantages of various analytical techniques used for the determination of organophosphorus and carbamate pesticides from aqueous and soil samples.

Table 1.3: Comparison of different analytical methods to determine pesticides [16]

Technique	Advantages	Disadvantages
GC	• High resolving power and ability to resolve individual analytes. • High sensitivity and good selectivity with element-selective detectors.	• Inadequate for polar, thermo – labile and low volatile compounds • High consumption of expensive, high – purity gases
GC/MS	• High resolving power and ability to resolve individual analytes • High sensitivity and selectivity • Existence of mass spectrum libraries for screening unknown samples	• Inadequate for polar, thermo – labile and low volatile compounds • High consumption of expensive, high- purity gases
LC-UV	• Application to virtually any organic solute, regardless of its volatility or	• Insufficient separation efficiency and selectivity

	thermal stability • Compositions of both mobile and stationary phase are variable • Can be automated and miniaturized (microchip technology) • Low price, simplicity, robustness and large linear range	• Large amounts of expensive, toxic, organic solvent used as mobile phase • Lack of matrix interferences
LC-Fluorescence	• High separation efficiency	• Few compounds are fluorescent
LC-MS	• Application to virtually any organic solute regardless to its volatility or thermal stability • Compositions of both mobile and stationary phase are variable • Can be automated and miniaturized	• Strongly affected by matrix interferences • Identification difficult using interfaces that provides soft ionization

1.4 Objectives of the research

The presence of pesticides in water and the food chain is a threat to human health. Determination of pesticides with conventional methods is time consuming, expensive and needs trained personal. Brennan's group, working under SENTINEL network, has developed a paper-based biosensor for determination of pesticides. This paper-based biosensor is a rapid, low cost device, to determine organophosphorus and carbamate pesticides in water and food. However, before technology transfer and market acceptance, these paper-based sensors have to be validated with field samples. As these biosensors was tested with fairly clean samples, with target analytes in solvent and buffer prepared using milli-Q water, in the laboratories it is developed.

The current research work intends to achieve the following objectives:

i. The main objective of this research is to validate the pesticide detecting paper-based biosensor with field samples under different environmental conditions and to correlate the results obtained with conventional methods.
    a. First goal is to study the activity of the enzyme and the shelf life of the strips.
    b. Secondly, the research is focused on identifying the possible interferences in field samples, which may inhibit the enzyme and give false positive result.
    c. Thirdly, we aim to study whether biosensors are capable of performing quantitative analysis.
ii. The generation 1 sensor suffers from a drawback of curling because of its length, and is not user friendly. In this work we aim to optimize the design to overcome the drawback while also making them user friendly, at the same time optimizing the fabrication of biosensors using commercially available office inkjet printer.
iii. Another objective is to conduct a matrix study, to observe the effectiveness of the paper-based biosensor to determine the presence of pesticides in coconut water.

2. MATERIALS AND METHODS

2.1 Reagents:

Acetylcholinesterase (AChE, from Electrophorus electricus, electric eel), Indoxyl acetate, and pesticides organophosphates (OPPs) (malathion, paraoxon, monocrotophos, dimethoate, parathion-methyl) were obtained from Sigma-Aldrich. Dichloromethane, diethyl ether, petroleum benzene (60-80 boiling range), HPLC grade methanol and sodium sulfate anhydrous GR were obtained from Merck. Florisil (60-100 mesh, pesticide residue analysis grade) and n-hexane (pesticide residue analysis grade Grade) were obtained from Finar. Distilled deionized water (HPLC grade) was obtained from Merck. Tris and acetone were obtained from SD Fine Chemicals and Rankem.

Solution Preparation:

Stock solution of IDA (100mM) was prepared using methanol and made up to IDA 10mM with methanol and tris buffer (100mM, pH 8) (50:50). AChE(250 U/ml) was prepared using tris buffer (100mM, pH 8). Stock solutions of malathion, paraoxon, monocrotophos, dimethoate and parathion-methyl were made up day-to-day and were not used for more than 3 h after preparation, to minimize the potential for degradation. A mixture of 5% methanol and tris buffer (10mM of pH 6.8) was used for dissolving malathion, paraoxon, monocrotophos, dimethoate and parathion-methyl was dissolved in dichloromethane. These solvents not only aided the solubility of AChE inhibitors, but do not have any effect on AChE activity, when used in lower concentration [37, 38].

2.2 Production of bioactive paper sensor for detection of pesticides:

The outlines of biosensor strips were printed with Whatman paper #1 using XEROX phaser 8560 wax printer. The printed papers were baked in the oven at 120^{o}C for two mins, this would melt the wax and create a hydrophobic barrier. Three layers of AChE (250U/mL) in 0.1% triton x-100 and tris buffer

(100mM, pH 8.0) and three layers IDA (10mM) in 50:50 methanol and tris buffer (100mM, pH 8.0) were printed in two different regions (sensing and substrate zone) using canon MP287 inkjet printer. Black cartridge was used to print bio-inks, black cartridge was emptied, cleaned and dried prior filling the bio-ink. Separate cartridges were used for printing enzyme and substrate, after printing bio-inks, cartridges were washed with water. Water or dyed water was printed with cartridges to inspect for clogging. Bio inks were modified with respect to surface tension and viscoscity using Triton X-100.

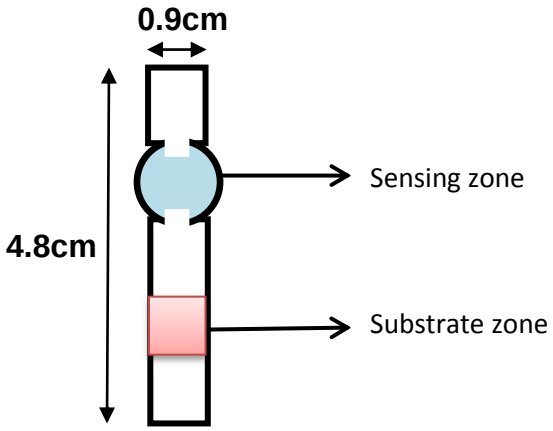


Figure 2.1: Pattern of pesticide detecting bioactive paper strip (0.9 X 4.8 cm) depicting sensing and substrate zones.

2.3 Measurement of Pesticides using paper-based biosensors:

The inhibitory effects of malathion, paraoxon, monocrotophos, dimethoate, parathion – methyl (organophosphates) pesticides were evaluated on the paper-based biosensor by measuring the decrease in color intensity produced by IDA–based colorimetric assay. The sensing region of the biosensor strip was incubated with varying concentrations (10mM to 1nM) of organophosphate pesticides solutions for 15 min. The substrate region of the strip was dipped into ddH_2O to move IDA to sensing region, to initiate enzyme catalyzed hydrolysis of substrate, results in development of blue color. Development of blue color indicates the absence of pesticide. No color or decrease in the blue color

intensity indicates the presence of pesticide. After drying, color intensity of sensors was determined by scanning the sensors using mobile scanner from Flip-Pal®, US. The color intensity was obtained by analyzing the digital image using ImageJ software (1.46r).

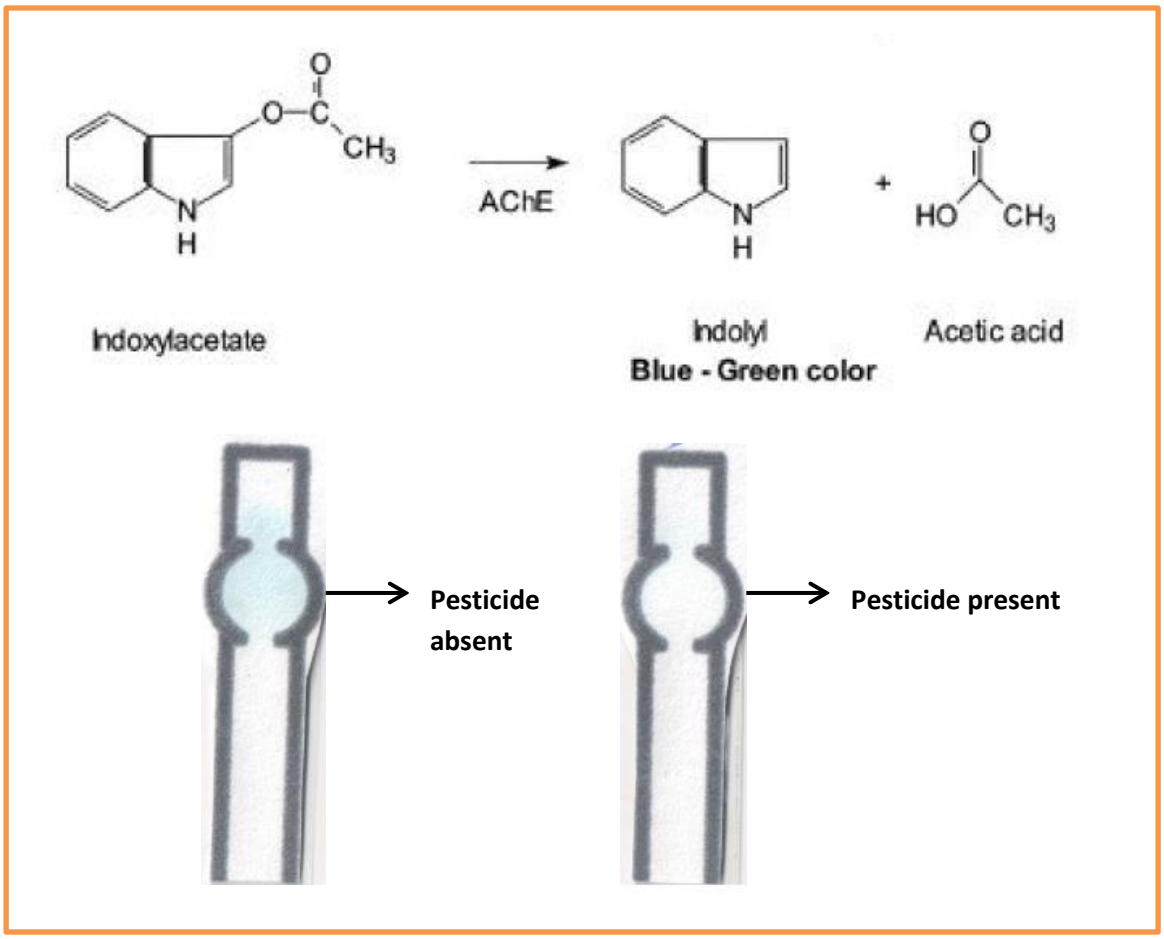


Figure 2.2: Example of paper-based sensor for detecting pesticides.

2.4 Sampling:

Water samples were collected from 12 sampling points from four villages. Surface water samples (Cauvery River and Kapila River) and ground water samples (from open wells and bore wells) were collected from different villages around Mysore city in southern India. Both surface water and ground water samples were collected in amber glass bottles and transferred from field to the

lab with ice pack. Bottles were rinsed with samples before collecting water. Samples were collected thrice from the same sampling points, once in fortnight in order to observe any variations with results. Samples were stored at 8^0C in the lab until extraction was done. Sample extraction (see section 2.6 for extraction procedure) was carried out within 72 hours of sampling. Extracted samples were stored at 4^0C until GC-MS analysis.

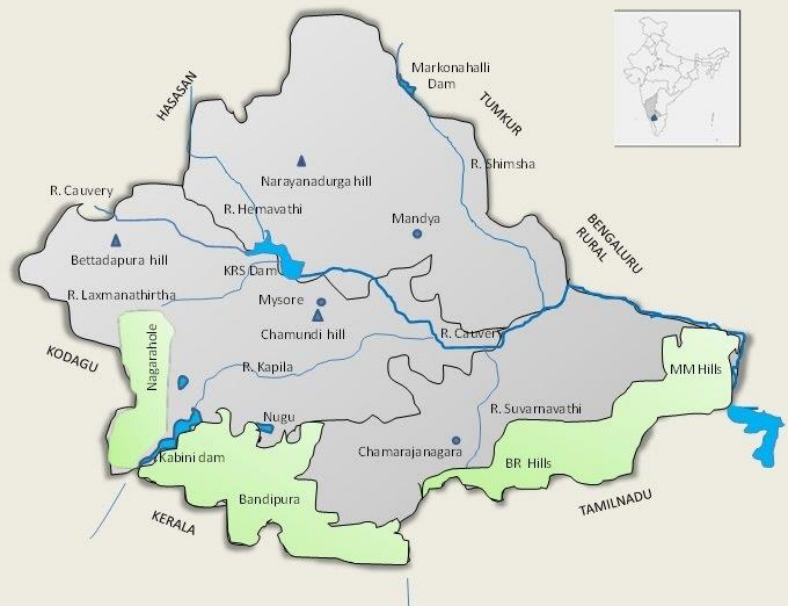


Figure 2.3: Mysore and surrounding villages map showing two main rivers, Cauvery and Kapila, the main source of surface water in this region.

2.5 Determination of pesticides in water samples using paper based biosensors:

Prior to analysis, water samples were filtered using 0.25 µm filter. Water samples were spotted in the sensing zone of the paper- based biosensors and incubated for 15mins. After the incubation, paper sensors were dipped in the samples to move the substrate to sensing zone as mentioned above. Wet strips were let to dry completely (15–20mins, depends on the surrounding temperature) and observed for stable blue color signal. Later strips were scanned using

portable scanner. Intensity of color was obtained by analyzing the digital image using ImageJ software. Color intensity of the color developed was compared with control strips for determination of presence of pesticides in water samples. No blue color or decrease in the blue color intensity indicates the presence of pesticides.

2.6 Determination of pesticides in water samples using GC-MS:

Extraction:

Liquid-liquid extraction with dichloromethane was used for extracting pesticide residues from water. 1000mL of water sample was extracted with 100mL of dichloromethane using a separating funnel, the procedure was repeated thrice, finally 300mL of dichloromethane containing pesticide residue was evaporated using rotary evaporator. The residue was diluted with petroleum benzene and diethyl ether mixture (85:15 v/v). This solvent mixture with pesticide residue was passed through glass column packed with Florisil (activated magnesium silicate, pesticide residue analysis grade) and anhydrous sodium sulfate. Florisil is a registered trade name of U.S. Silica Co. for magnesium silicate, it is used to separate analytes from interfering compounds, and anhydrous sodium sulfate helps in trapping moisture. Eluted solvent was evaporated and residue was dissolved in n-hexane. Pesticide residue in n-hexane was injected to GC-MS for analysis.

Instruments:

Analysis of organophosphate and carbamate pesticides were performed using a Agilent Technologies 7890A GC with Agilent Technologies 5975C MS system equipped with 30 m x 0.320 mm DB–35MS (35%phenyl – methylpolysiloxane) fused silica mid polar capillary column with 0.25 µm film thickness (Agilent J & W, USA). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The injector and thermal auxiliary 2 (MSD transfer line) were

operated at 250^{o}C and 280^{o}C, respectively. The operation conditions were: acquisition-mode scan and SIM (selective ion monitoring), with gain factor 10 (2470 V). Sample (2 µl) was injected in the splitless mode, and the oven temperature was programmed as follows: 40 °C for 0 min, ramping to 150 °C (20 °C/min) for 0 min, to 175 °C (5 °C/min) for 0 min, to 195 °C (2 °C/min) for 0 min and to 300 °C (10 °C/min) and held for 5 min. Table 2.1 shows the spectral characterization using GC-MS with electron impact ionization (EI) mode of the pesticides studied.

Table 2.1: Spectral characterization using GC-MS with (EI) of the pesticides studied.

Pesticides	***m/z* (Target ion)**	**Fragment ions**	**Rt (Retention time) mins**
Malathion	173	93,158,285,256	21.38
Dimethoate	93	87,125,229	16.39
Paraoxon	109	149,139,275	20.61
Carbofuron	164.1	221,149	16.23
Methyl Parathion	109	125,263	20.48

2.7 Assessing effect of water samples containing commercial pesticides on assay performance:

Commercial pesticides usually contain 30 – 60% of pesticides and remaining will be solvent and emulsifiers to maintain the stability. In order to assess the performance of paper-based sensor to determine these commercial pesticides in water samples under different environmental conditions, surface water and ground water were spiked with varying concentrations of commercially available malathion and dimethoate. (10mM – 1nM). Prior to spiking the water samples with pesticides, samples were analysed using GC-MS to confirm the absence of pesticides. The measurements were conducted with paper-based sensors as explained above.

2.8 Analysis of monocrotophos in coconut water:

The matrix effect in the analysis of monocrotophos in coconut water was studied. Tender coconuts from same tree were collected from the field, coconut water sample was filtered using 0.25µm filter. Commercially available monocrotophos was mixed in 100mL coconut water (6.5 pH) to obtain final concentration of 1mM and further dilutions were made to obtain varying concentrations (10mM – 1nM). Coconut water sample was tested using paper-based sensor to determine the pesticide residue, sensors were scanned using Flippal portable scanner after drying, in order to determine color intensity. The color intensity was obtained by analyzing the digital image using ImageJ software. Prior to spiking the coconut water sample with pesticides, sample was analyzed using GC-MS to confirm the absence of pesticides. Sample preparation for GC-MS analysis was done using liquid-liquid extraction with dichloromethane, in the similar procedure explained above for water sample. GC-MS instrument parameters were retained same as used for water samples.

3 RESULTS

In this section, we are reporting the issues with shipment of the paper-based sensors. Information regarding the printing and storage stability of the sensor is explained. Results obtained from the evaluation of paper-based sensors with water samples from different aquatic environment along with validation data using GC-MS are summarized.

3.1 Importing Strips

Initially the paper-based biosensors were fabricated in Canada and shipped to India for the evaluation study. Biosensors were fabricated by printing both AChE (enzyme) and substrate (IDA) in between two layers of silica, and a layer of capturing agent (poly arginine) was printed under the silica layer in enzyme region, this helps in capturing the color [13]. Strips were shipped with dry ice in order to keep the enzyme and substrate viable. When strips were received, we observed development of pink color in the substrate region, which indicated that the substrate (IDA) was oxidized and was not viable. Presumably the IDA was oxidized due to temperature fluctuations during the shipment and storage before the customs clearance. This data show that silica layers do not help to maintain the stability of IDA. The activity of the enzyme on the imported biosensor was tested by pipetting IDA on the enzyme region; appearance of blue color indicated the activity of enzyme. This data demonstrate that, immobilization of enzyme (AChE) in between silica layer benefits in keeping the enzyme active inspite of temperature fluctuations during storage and shipment.

To overcome this issue, we imported wax outline printed Whatman#1 paper sheets. The strips were produced in India by printing enzyme and substrate without the silica and capturing agent using inkjet office printer (MP287, Canon). The biosensor strips were stored at 4^0C in order to keep the enzyme and substrate viable. The sensor performed similarly but suffered from a

drawback of leaching of blue color. This show that, capturing agent is required to hold the color.

3.2 Printing and storage stability of the sensor:

Generation 1 biosensor looks similar to litmus paper and was suffering from drawback of curling because of its length (8 cm). With this design of sensors it is difficult to identify the substrate and sensing zone for a common man and thus it was not user friendly. With modified design generation 2 biosensor overcomes this setback as the length is reduced by about half, and it does not have any impact on the performance of the strip. This version of sensor is more user friendly as sensing region and substrate region can be easily identified with the new pattern of the sensor and also the run time is reduced with decrease in length of the sensor (see figure 3.1).

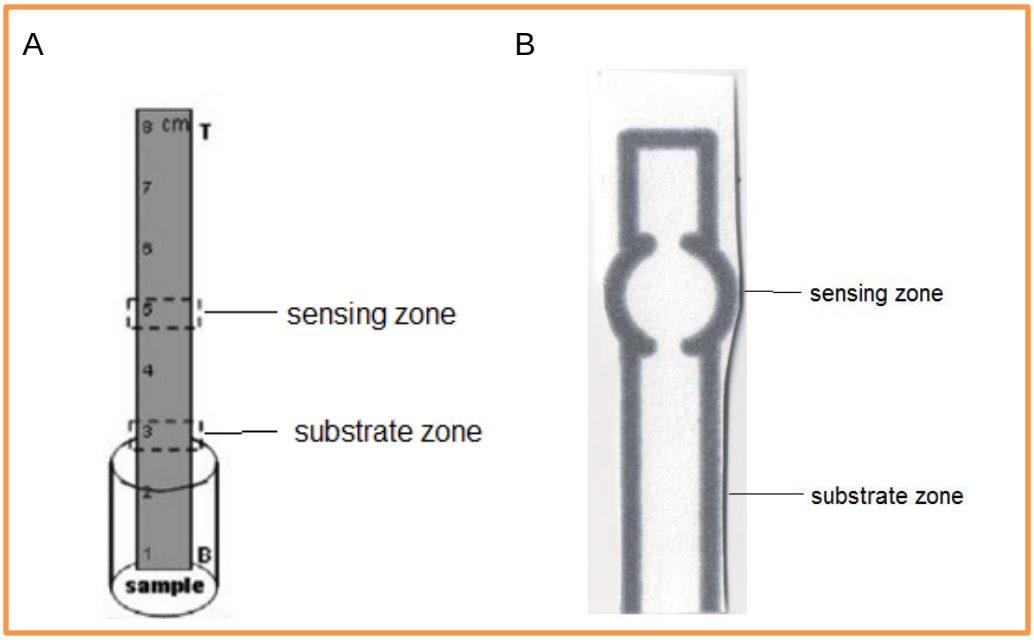


Figure 3.1: example of generation 1 biosensor (A) and generation 2 biosensor (B) showing sensing and substrate regions.

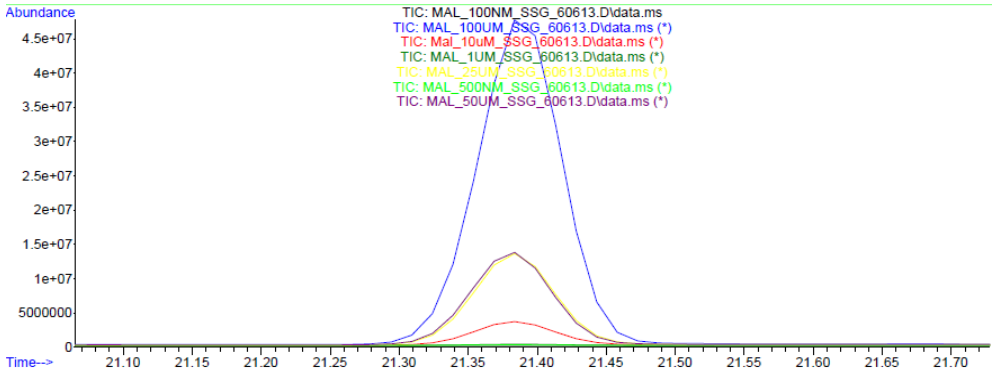
Further, though we are successful in printing the bioinks using inkjet office printer (MP287, Canon), the cartridge gets clogged frequently, in case of AChE printing, usually cartridge gets completely clogged after printing 3-4 sheets. In case of IDA printing, cartridge gets completely clogged after printing 6 – 9 sheets. But at times, cartridge would get clogged after single use with both AChE and IDA. We have to overcome the issue of clogging of the cartridge, in order to make the fabrication efficient and cost effective. Another major issue with printing the bioinks with inkjet printer is we do not know the volume of the bioinks printed on each sensor. As we do not have any hold on the volume of the bioinks printed on the paper support, there may be chances of variations in volume of bioink printed on sensors of the same batch. The difference in volume of bioinks printed can be identified by measuring the color intensity after running the blank test with distilled water. These observations indicate that, fabrication of biosensor strips needs further optimization and care should be taken to maintain the consistency with the volume of the bioink printed. Our observation shows that both enzyme and substrate printed without the silica layer were viable for about one month when stored at 4^{o}C. Entrapping AChE with silica supports AChE to retain its activity inspite of variation in the storage temperature during shipment. Entrapping IDA with silica will not help in retaining its stability during the fluctuations in the temperature. Thus further optimization is essential to stabilize IDA to withstand the temperature variations to allow the shipping of biosensors.

3.3 Determination of pesticides in water samples and coconut water using GC-MS.

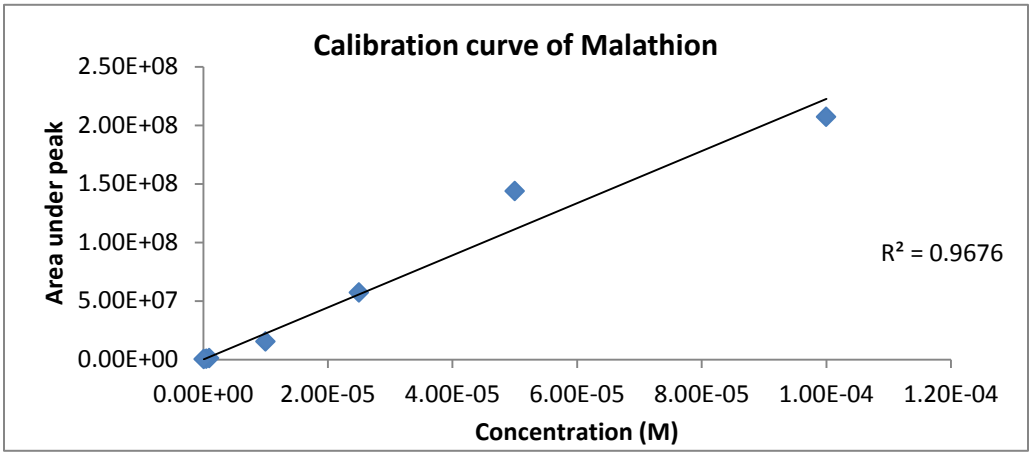
In this study, liquid-liquid extraction combined with GC-MS method was developed for determination of organophosphorus pesticides in water samples and coconut water. Linear calibration curves of different pesticides were obtained with the method developed, by using varying concentrations of samples of pure pesticides. The sensitivity of detection of instrument varied for different

pesticides. Lowest concentrations that could be detected with instrument used, for different pesticides is as follows: malathion-100nM, paraoxon-1µM, dimethoate-1µM, methylparathion-100nM. This method was further used for qualitative analyses of water samples and coconut water to determine the presence of analytes, and to further validate the results obtained from biosensor.

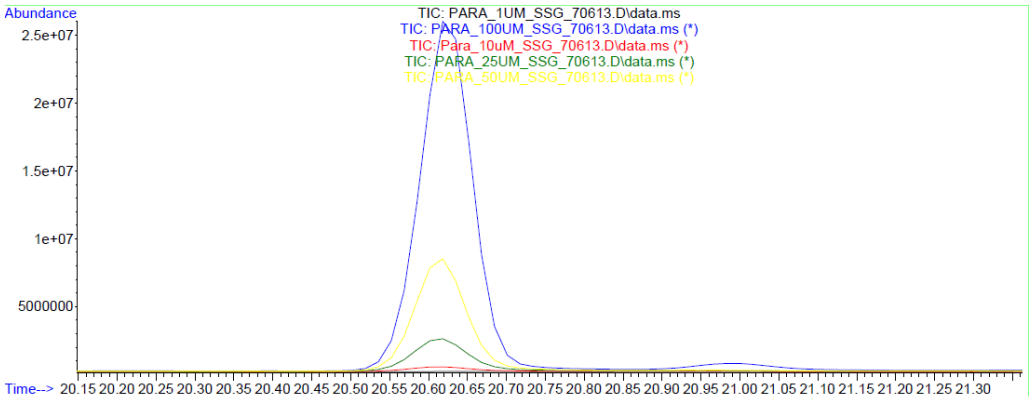
A



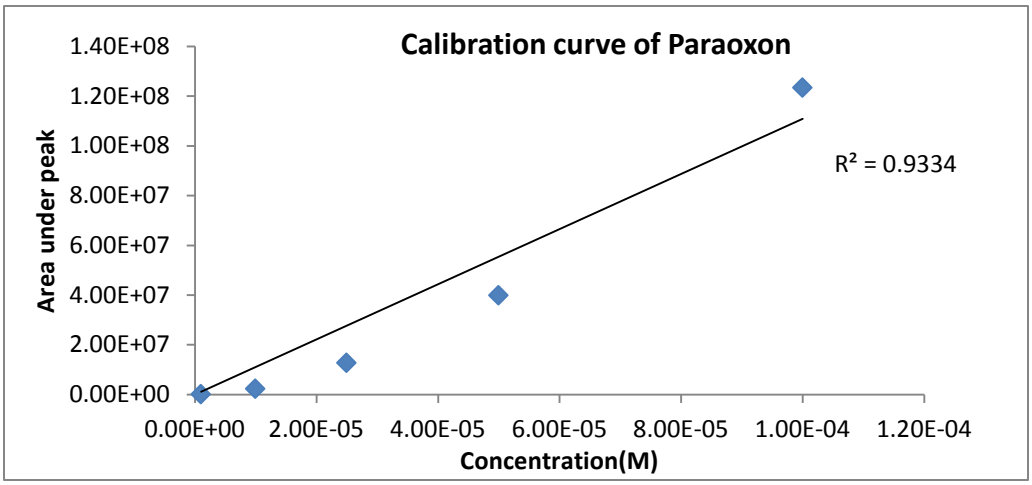
a



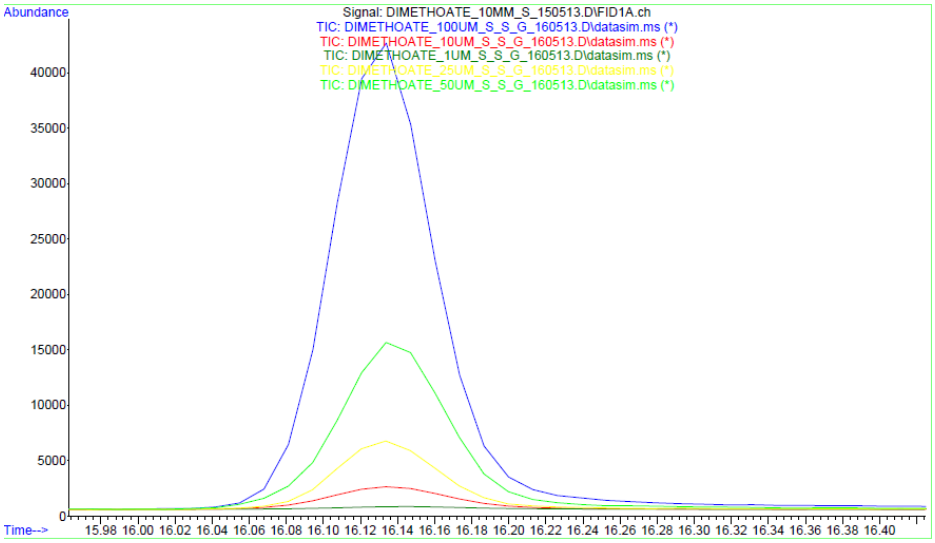
B


Abundance
TIC: PARA_1UM_SSG_70613.D\data.ms
TIC: PARA_100UM_SSG_70613.D\data.ms (*)
TIC: Para_10uM_SSG_70613.D\data.ms (*)
TIC: PARA_25UM_SSG_70613.D\data.ms (*)
TIC: PARA_50UM_SSG_70613.D\data.ms (*)
2.5e+07
2e+07
1.5e+07
1e+07
5000000
Time--> 20.15 20.20 20.25 20.30 20.35 20.40 20.45 20.50 20.55 20.60 20.65 20.70 20.75 20.80 20.85 20.90 20.95 21.00 21.05 21.10 21.15 21.20 21.25 21.30

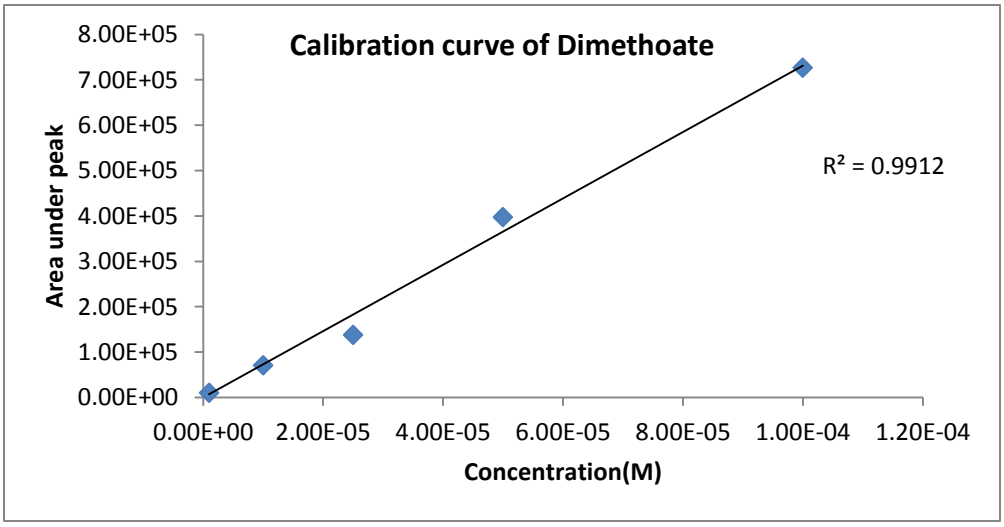
b


Calibration curve of Paraoxon
Area under peak
1.40E+08
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
R² = 0.9334
0.00E+00 2.00E-05 4.00E-05 6.00E-05 8.00E-05 1.00E-04 1.20E-04
Concentration(M)

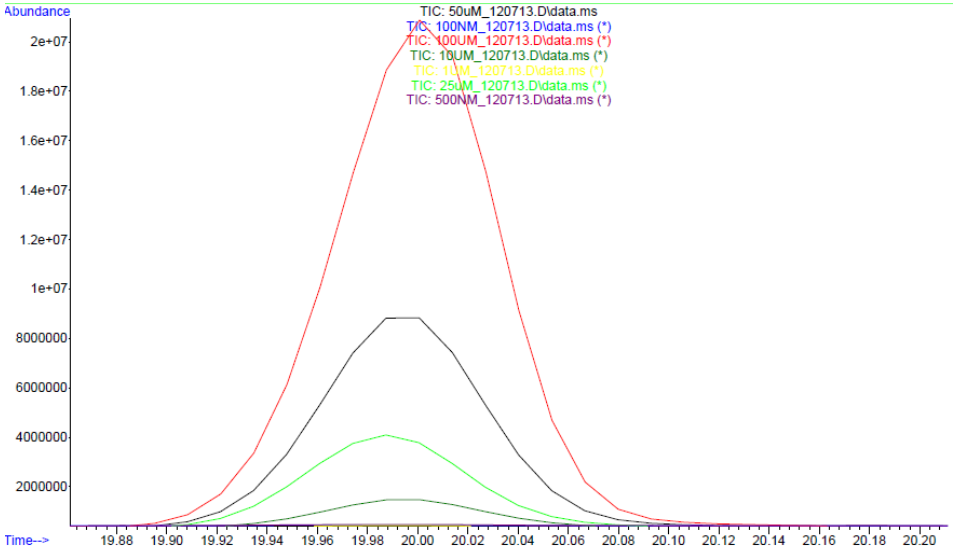
C


Abundance
Signal: DIMETHOATE_10MM_S_150513.D\FID1A.ch
TIC: DIMETHOATE_100UM_S_S_G_160513.D\datasim.ms (*)
TIC: DIMETHOATE_10UM_S_S_G_160513.D\datasim.ms (*)
TIC: DIMETHOATE_1UM_S_S_G_160513.D\datasim.ms (*)
TIC: DIMETHOATE_25UM_S_S_G_160513.D\datasim.ms (*)
TIC: DIMETHOATE_50UM_S_S_G_160513.D\datasim.ms (*)
40000
35000
30000
25000
20000
15000
10000
5000
0
Time--> 15.98 16.00 16.02 16.04 16.06 16.08 16.10 16.12 16.14 16.16 16.18 16.20 16.22 16.24 16.26 16.28 16.30 16.32 16.34 16.36 16.38 16.40

c


Calibration curve of Dimethoate
Area under peak
8.00E+05
7.00E+05
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
R² = 0.9912
0.00E+00
2.00E-05
4.00E-05
6.00E-05
8.00E-05
1.00E-04
1.20E-04
Concentration(M)

D


Abundance
TIC: 50uM_120713.D\data.ms
TIC: 100NM_120713.D\data.ms (*)
TIC: 100UM_120713.D\data.ms (*)
TIC: 10UM_120713.D\data.ms (*)
TIC: 1UM_120713.D\data.ms (*)
TIC: 25UM_120713.D\data.ms (*)
TIC: 500NM_120713.D\data.ms (*)
2e+07
1.8e+07
1.6e+07
1.4e+07
1.2e+07
1e+07
8000000
6000000
4000000
2000000
Time-->
19.88
19.90
19.92
19.94
19.96
19.98
20.00
20.02
20.04
20.06
20.08
20.10
20.12
20.14
20.16
20.18
20.20

d

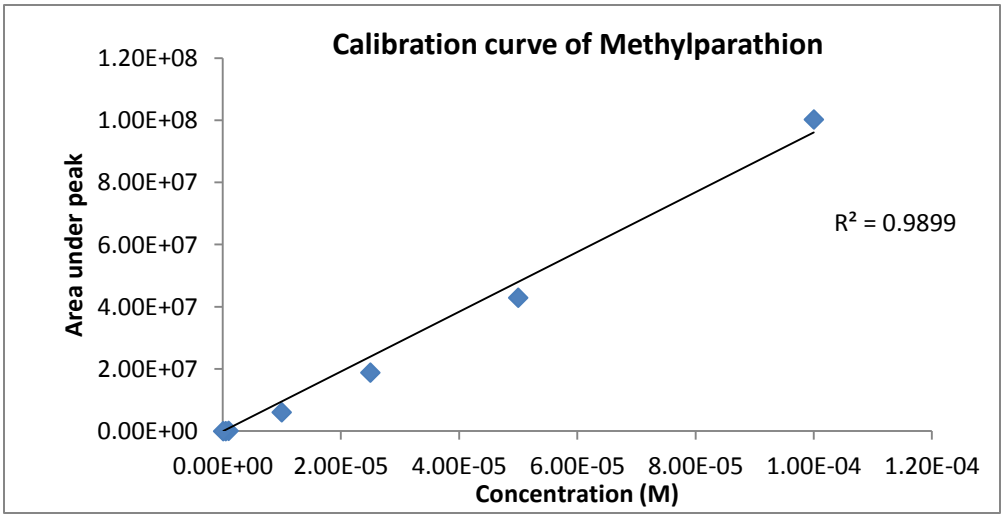

Calibration curve of Methylparathion
Area under peak
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
R² = 0.9899
0.00E+00
2.00E-05
4.00E-05
6.00E-05
8.00E-05
1.00E-04
1.20E-04
Concentration (M)

Figure 3.2: chromatogram of different pesticides displaying the linearity with varying concentrations and calibration curves of pesticides. A-a show the chromatogram and calibration curve of malathion, B-b show the chromatogram and calibration curve of paraoxon, C-c show the chromatogram and calibration curve of dimethoate, D-d show the chromatogram and calibration curve of methylparathion.

3.4 Sensitivity of paper-based biosensor for determination of different pesticides:

The outlines of biosensor strips were printed with Whatman paper #1 using wax printer. The printed papers were baked in the oven at 120^{o}C for two mins, this would melt the wax and create a hydrophobic barrier. Three layers of AChE (250U/mL) in 0.1% triton x-100 and tris buffer (100mM, pH 8.0) and three layers IDA (10mM) in 50:50 methanol and tris buffer (100mM, pH 8.0) were printed in two different regions (sensing and substrate zone) using inkjet printer.

A survey was conducted in the field of study, by interviewing the local people in the field and also in the commercial outlets to know the extensively used pesticides in this area. Based on the survey, top five (paraoxon, malathion, dimethoate, monocrotophos and methyparathion) extensively used organophosphorus pesticides in the field of study were selected as analytes of interest. Under laboratory conditions, analysis was performed to ascertain the sensitivity of the paper-based sensor in detecting the lower concentration of these pesticides. The measurements were performed by incubating the biosensor strips with different concentrations of analytes for 15min and then substrate (IDA) was moved to the sensing zone by dipping substrate region of the biosensor strip in the ddH_2O to initiate enzyme catalyzed hydrolysis of substrate, results in development of blue color. Incubation time plays a vital role in this assay, increased incubation time allows the toxins to penetrate through pores of paper surface and inhibit enzyme. Biosensor strips were incubated with known volume of toxins, for different time intervals from 5mins to 20 mins, we observed the difference in color intensity with incubation time. The biosensor

strips were scanned using flippal portable scanner to obtain the digital image, these images were analyzed using imgeJ software to acquire color intensity of the sensors. As there was not much difference in color intensity between 15mins and 20mins incubation (see figure 3.3), we conducted all the assays by keeping 15mins as optimal incubation time.

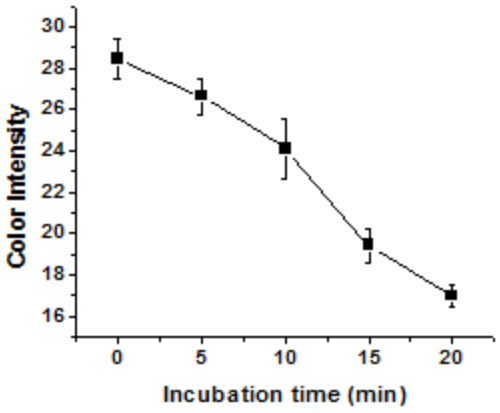
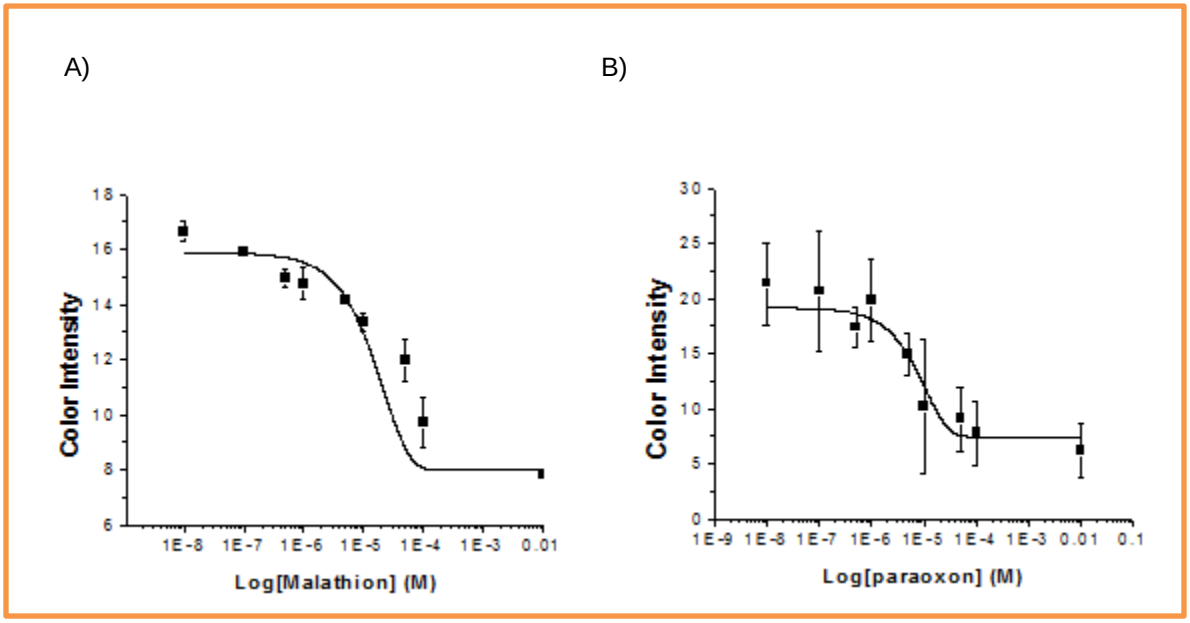


Figure 3.3: graph demonstrating effect of incubation time on intensity of the color developed. All points are mean values of three independent measurements.



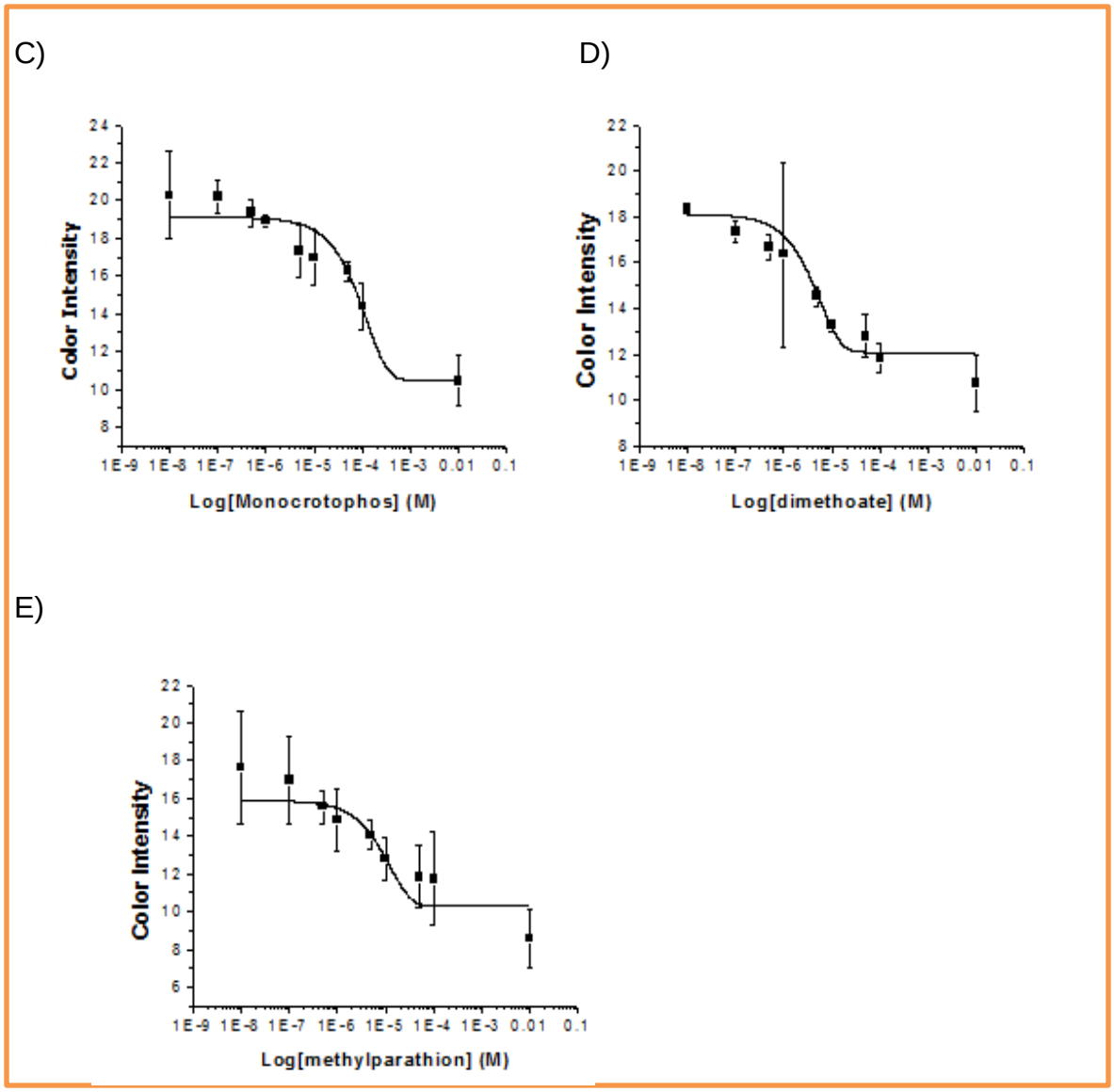


Figure 3.4: dosage-based inhibition of acetylcholinesterase (AChE) enzyme by different concentrations of organophosphorus pesticides. A-B) shows the dose-based inhibition response of malathion and paraoxon respectively. C-D) show the dose-based inhibition response of monocrotophos and dimethoate respectively and E) show the dose-based inhibition response of methylparathion. All points are means ± s.d. of three independent measurements for each concentration.

The inhibitory effects of malathion, paraoxon, monocrotophos, dimethoate, parathion–methyl (organophosphates) pesticides were evaluated on the paper-

based biosensor by measuring the decrease in color intensity produced by IDA–based colorimetric assay. The sensing region of the biosensor strip was incubated with varying concentrations (10mM to 1nM) of organophosphate pesticides solutions for 15 min. The substrate region of the strip was dipped into ddH_2O to move IDA to sensing region, for analyzing the performance of the biosensor strips. Development of blue color indicates the absence of pesticide. No color or decrease in the blue color intensity indicates the presence of pesticide.

Figure 3.4, A-E shows the inhibition response of acetylcholinesterase enzyme with varying dosage of malathion, paraoxon, monocrotophos, dimethoate and methylparathion respectively. The graphs are plotted with the mean value of color intensity from three independent measurements, and the errors in analyzing color intensity is due to variation in pixel area during image analysis using imageJ software. Our data show that AChE is inhibited progressively with increasing concentration of pesticides. We were able to make out the difference in color intensity upto 50nM for all the pesticides we tested, with naked eye.

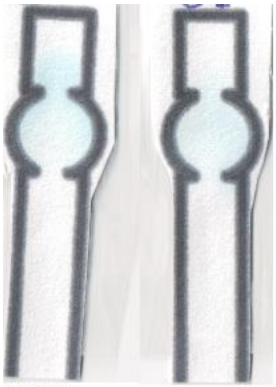
3.5 Performance of paper-based biosensor for detecting pesticide in water samples:

Though pesticides are banned in most developed countries, they are still being used extensively in developing countries. Both published work [13] and the results from the last section suggest that our paper-based sensors were capable of determining pesticides in water and food samples under optimal conditions in the laboratory . To assess whether our paper-based sensors were able to detect these toxic compounds in the environment under different environmental conditions, water samples from twelve sampling points were analyzed with our paper-based sensors. The samples collected were from both ground water and surface water sources. The sensing region of the paper-based biosensor was

incubated with water sample for 15mins, after the incubation, substrate end of the biosensor was dipped in the water sample, in order to move substrate (IDA) to sensing zone for analyzing the performance of the biosensor strips. The sensors were allowed to dry at room temperature until the stable color was developed. The strips demonstrated the presence of pesticides in water samples from three of the sampling points among the twelve sampling points. As the biosensor strips indicate only the presence or absence of the organophosphorus or carbamate pesticide, these samples were analyzed using GC-MS in order to validate the results obtained from biosensor strips. The data obtained from GC-MS shows the presence of methylparathion in these water samples. The results of water samples of both surface water and ground water that contained methylparathion, from both the methods are summarized below.

Surface water

a



b

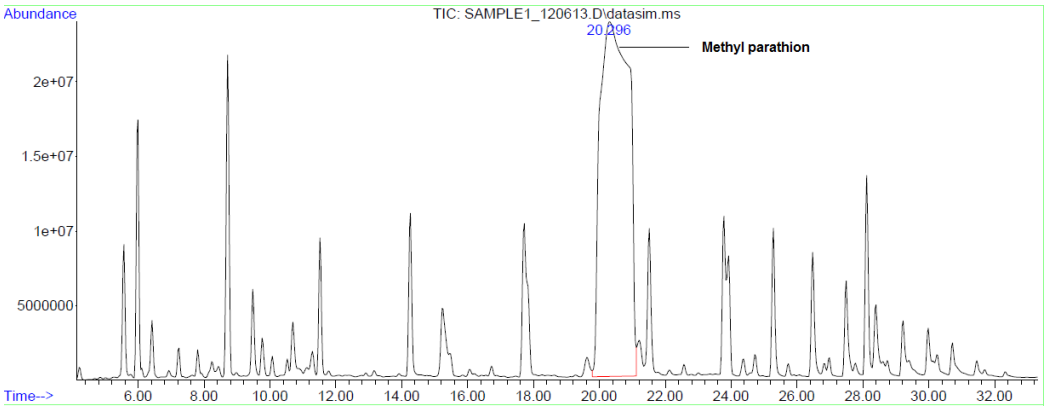


Figure 3.5: **a)** show the result of the analysis of surface water sample from biosensor strip, reduction in the color intensity compared to the control indicates the presence of pesticide. **b** show the result from GC-MS, depicting the chromatogram indicating the presence of methylparathion.

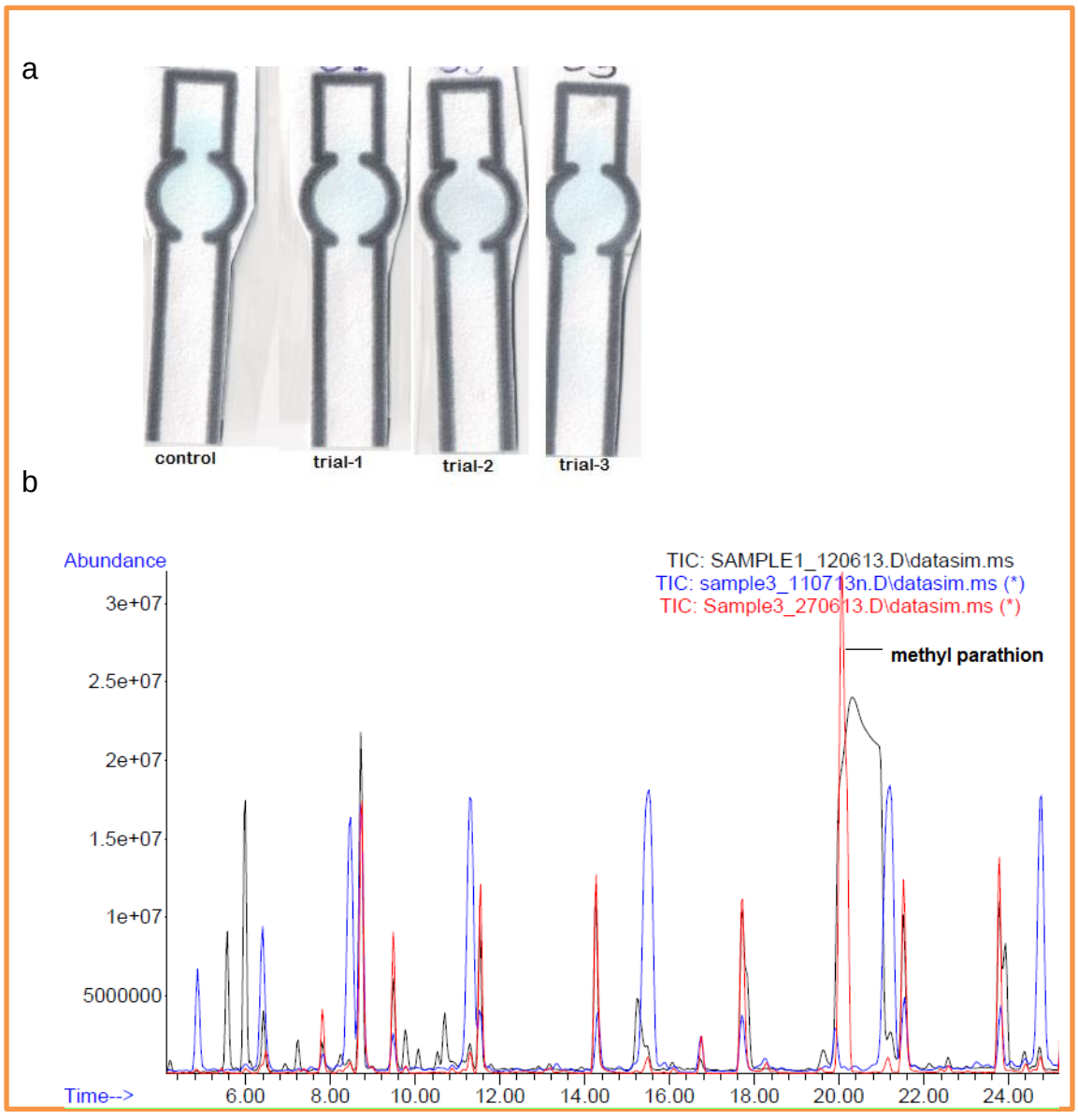


Figure 3.6: determination of pesticides in surface water with time (three trials, sampling done once in fortnight). **a**) show the results from biosensor and **b**) show the result from GC-MS.

Table 3.1: Data obtained from analysis of surface water samples with paper-based sensors and GC-MS analysis.

Surface water	**Color intensity (Biosensor strip data)**		**Area under peak (GC-MS data)**	**Concentration of methyl parathion (calculated from GC-MS data)**	**Maximum residual limit (MRL)**	
	Control	**Sample**			**IS:10500**	**WHO**
Trial 1	27.52	14.63	2147483647	5.15mM	1.13nM	Not defined
Trial 2	27.01	17.99	2147483647	5.15mM		
Trial 3	27.72	27.58	Not detected			

Both surface water and ground water was analysed for determination of pesticide using paper-based sensors and GC-MS. The result of one of the sampling point of surface water which contained pesticides is depicted in figure 3.5. In case of biosensor, the decline in color intensity with respect to control indicates the presence of pesticide. The same sample was analyzed using liquid-liquid extraction combined with GC-MS, analysis indicated the presence of methylparathion. The results obtained from both the methods indicated the presence of pesticide in this water sample. Performance of the paper-based biosensor with time was evaluated by conducting repetitive assay of the same samples with time interval of fifteen days. Figure 3.6 shows the comparison of the results of three independent samples of same sampling point, a) show the results from sensors and b) illustrate the results from GC-MS. The color intensity obtained from image analysis of paper-based assay and area under peak acquired from GC-MS analysis is tabulated in table 3.1. Our data obtained from biosensor and GC-MS indicates presence of toxin in first two trials and the results were negative in third trial. The data acquired from GC-MS confirms the results obtained from paper-based sensor, this indicates potential for using biosensor strips for rapid analysis of water samples for detection of pesticides in water sample.

Ground Water

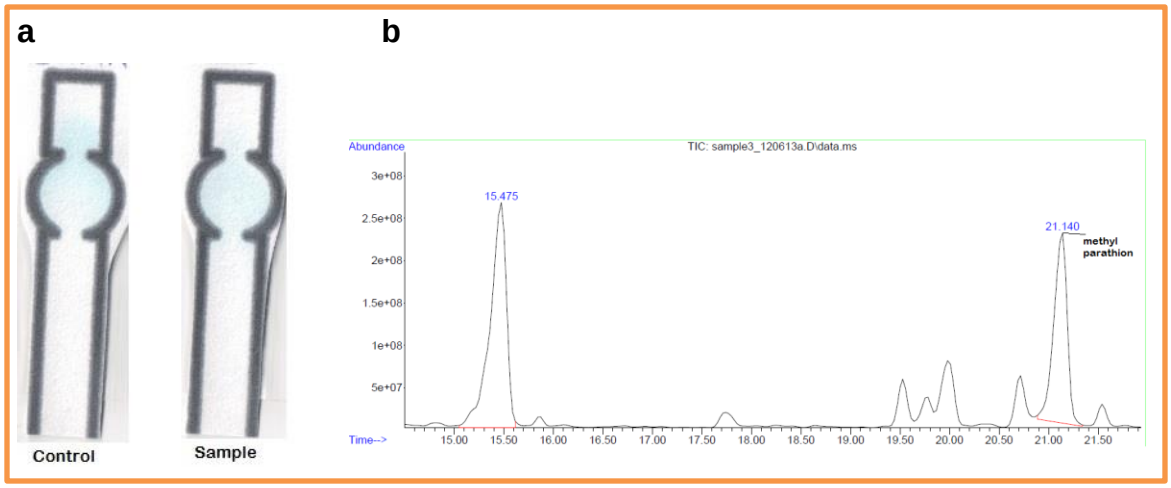


Figure 3.7: determination of pesticides in ground water. **a**) show the results from biosensor and **b**) show the result from GC-MS.

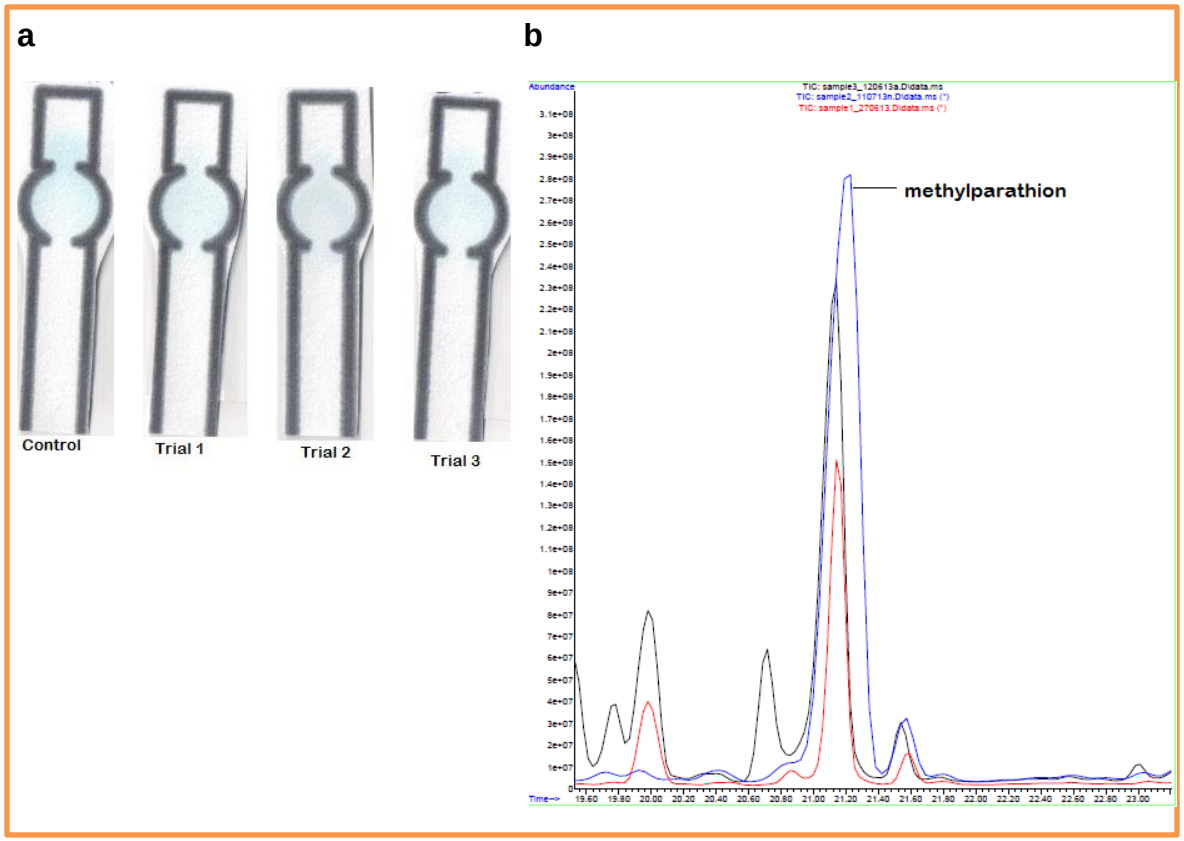


Figure 3.8: determination of pesticides in ground water with time (three trials, sampling done once in fortnight). **a)** show the results from biosensor and **b)** show the result from GC-MS

Table 3.2: Data obtained from analysis of ground water samples with paper-based sensors and GC-MS analysis.

Ground water	Color intensity (Biosensor strip data)		Area under peak (GC-MS data)	Concentration of methyl parathion (calculated from GC-MS data)	Maximum residual limit (MRL)	
	Control	Sample			IS:10500	WHO
Trial 1	27.52	16.39	555677884	559 µM	1.13nM	Not defined
Trial 2	27.01	21.34	11542621	14.54 µM		
Trial 3	27.72	19.71	22896502	25.89 µM		

Figure 3.7 shows the results of ground water assay from one of the sampling point which contained pesticide. The tests were conducted with the same method as described above. Our data from both methods are in agreement with each other. Evaluation study was conducted to assess the performance of biosensor with time. Samples were collected from the same sampling point thrice, with interval of fortnight, and analyzed for the detection of analytes of interest using both methods. Figure 3.8 shows the comparison of the results of three trials, a) shows the results from sensors and b) illustrates the results from GC-MS. Our data from the biosensors and GC-MS indicates presence of pesticide in all trials. The color intensity obtained from image analysis of paper-based assay and area under peak acquired from GC-MS analysis is tabulated in table 3.2. The data obtained from both the methods are in agreement with each other. The results indicate potential for using biosensor strips for rapid analysis of water samples for detection of presence of organophosphorus pesticide.

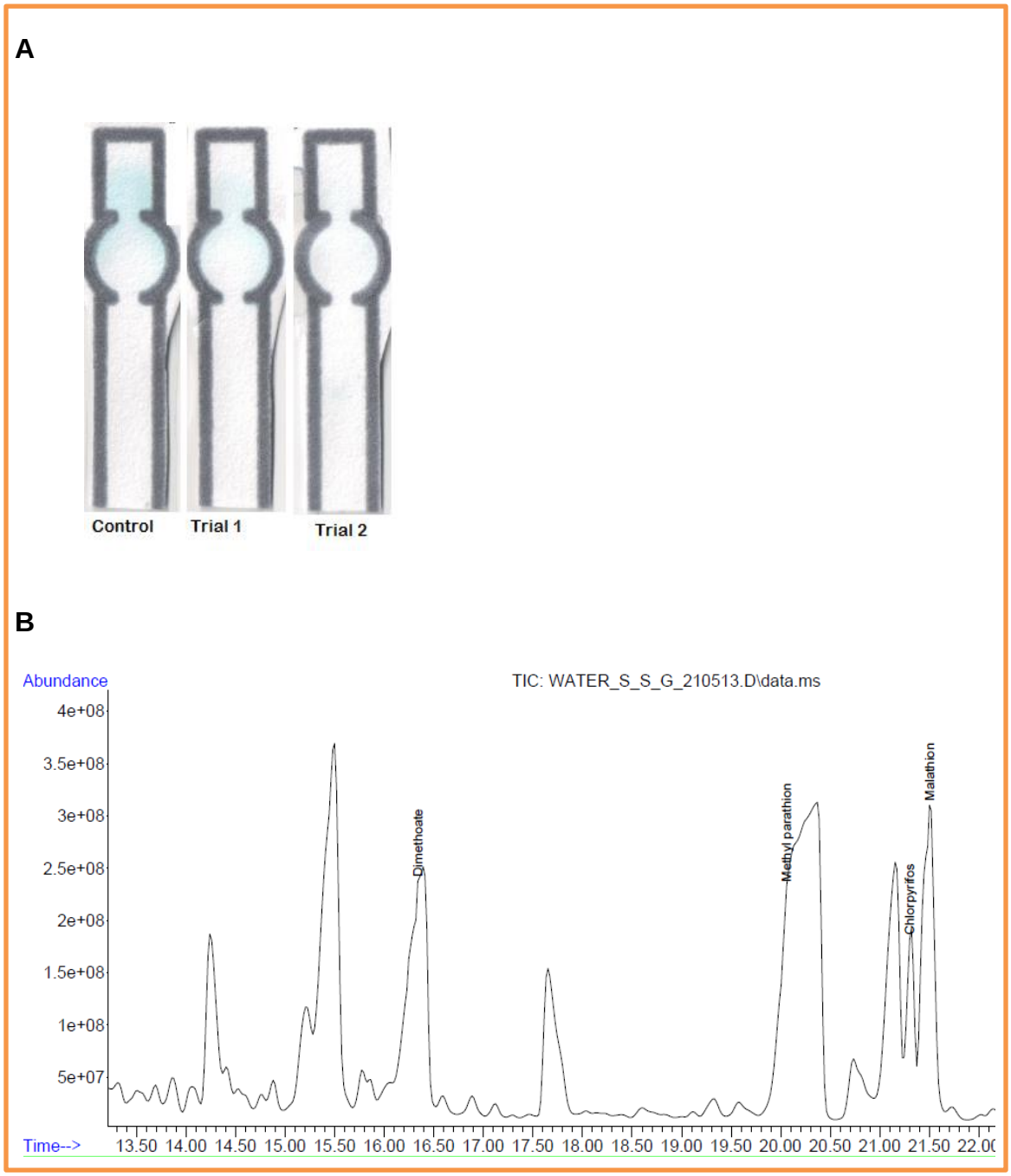


Figure 3.9: determination of pesticides in tap water (source is ground water). **a**) show the results from biosensor and **b**) display the result from GC-MS, chromatogram indicates the presence of dimethoate, methylparathion, chloropyrifos and malathion .

Table 3.3: Data obtained from analysis of water sample with paper-based sensors and GC-MS analysis.

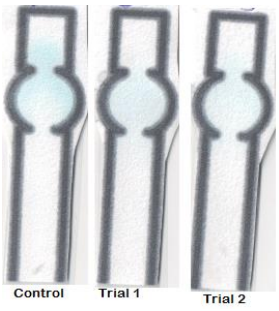
Pesticides in Water Sample	**Color intensity (Biosensor strip data)**		**Retention time (GC-MS data)**	**Concentration (calculated from GC-MS data)**	**Maximum residual limit (MRL) IS:10500**
	Control	**Sample**			
Dimethoate	27.686	12.453 (mean value of three independent measurements)	16.380	227µM	Not defined
Methyl Parathion			20.138	517µM	1.13nM
Chlopyrifos			21.068		85.57nM
Malathion			21.506	250 µM	575nM

Figure 3.9 shows the results of a tap water sample (ground water source) which contained more than one pesticide. When this water sample was analysed with the paper-based biosensor, color intensity decreased tremendously with respect to control sensor. The chromatogram from the GC-MS analysis indicated the presence of dimethoate, methylparathion, chloropyrifos and malathion. The color intensity obtained from an image analysis of paper-based assay and area under peak, retention time of different pesticides present in the sample acquired from GC-MS analysis are tabulated in table 3.3. From these data, we infer that biosensors are able to detect presence of pesticides either alone or in mixture. Paper-based biosensors can be used for only qualitative analysis of samples for the detection of pesticide, as strips can only indicate the presence or absence of pesticides. Further analysis using conventional method is required to identify the pesticides and their concentration.

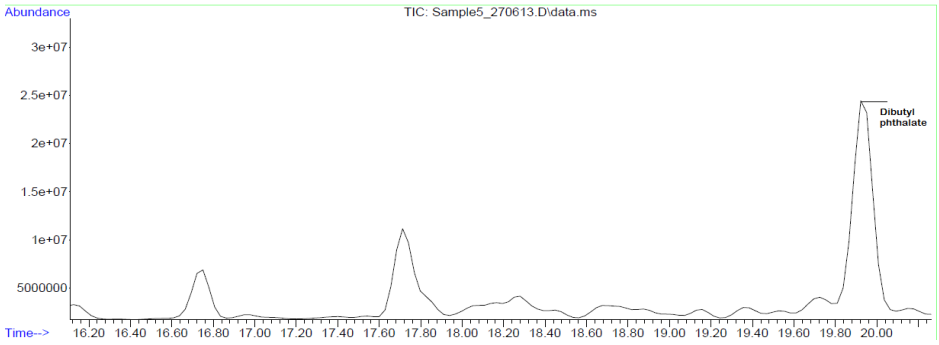
3.6 Possible interferences for paper-based biosensor assay to sense pesticides in water samples.

One of the objectives of our study was to identify probable interferences in the aquatic environment, which can interfere with the assay. With few water samples among twelve sampling points, we observed difference in the result from paper-based sensor and GC-MS. Generally, GC-MS analysis indicated the presence of dibutyl phthalate in all these samples (Figure 3.10). Though acetylcholinesterase is considered as the biomarker for indicating the presence of organophosphate and carbamate pesticide in aquatic environment, J.C. Kang and team, Pukyong National University, Korea has reported that dibutyl phthalate and di-ethylhexyl phthalate inhibit acetylcholinesterase [39]. Dibutyl phthalate and di-ethylhexyl phthalate are extensively used plasticizers to render flexibility to plastic products. Since these plasticizers are loosely bound to plastic mediums, they easily leach out to their surrounding environment. We tested our paper-based sensor with dibutyl phthalate, the data showed that dibutyl phthalate inhibit AChE, and extent of inhibition is more with higher concentration (above 100µM).

a



b



c

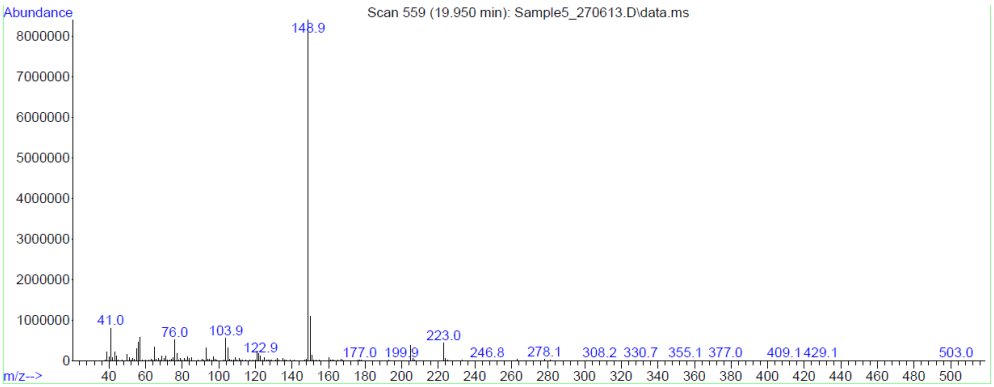


Figure 3.10: **a**) example of dibutyl phthalate present in water sample inhibiting AChE in biosensor. **b**) Chromatogram of water sample displaying the presence of dibutyl phthalate, **c**) mass spectra of dibutyl phthalate

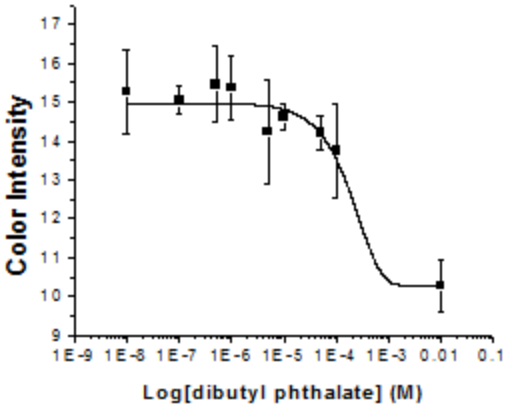


Figure 3.11: dose-based inhibition of acetylcholinesterase enzyme by different concentrations of dibutyl phthalate.

Figure 3.11 demonstrates the dose-based inhibition of acetylcholinesterase enzyme by different concentrations of dibutyl phthalate. The biosensor strips were incubated with varying concentrations (10nM to 10mM) of samples of pure dibutyl phthalate for 15 mins. The substrate region of the strip was dipped into ddH_2O to move IDA to sensing region, for analyzing the performance of the biosensor strips. The decrease in color intensity or no color indicated the inhibition of AChE by dibutyl phthalate. Not much difference in color

intensity was observed with color intensity obtained from image analysis between the lower concentrations (10nM to 50µM) of dibutyl phthalate. But assessable difference in color intensity was observed with naked eye in case of higher concentration (in the range between 100uM to 10mM) of dibutyl phthalate. Our data show that dibutyl phthalate inhibit acetylcholinesterase, from this we can infer that presence of high concentration (100uM to 10mM) of dibutyl phthalate in water samples can interfere with paper-based biosensor assay for the determination of pesticides.

3.7 Assessing effect of water samples containing commercial pesticides on assay performance:

Pesticides used in agriculture, industry which enters the aquatic environment are commercially available pesticides. These commercial pesticides usually contain 30 – 60% of pesticides and remaining will be solvent and emulsifiers to maintain the stability. Paper-based biosensors were tested for its performance in the laboratory with standard pesticides samples (99% pure), by dissolving pure pesticides in solvent and buffer (prepared using milli-Q water) to maintain the pH. In order to assess the performance of paper-based sensor to determine the commercial pesticides in field water samples, surface water and ground water samples were collected from the field and spiked with varying concentrations of commercially available malathion and dimethoate (10mM – 10nM). The water samples were analysed using GC-MS to confirm the absence of pesticides in these water samples. No pH adjustments were done, pH of surface water was 7 and pH of ground water was 8. The inhibition pattern of standard solutions and commercial pesticides in water was studied.

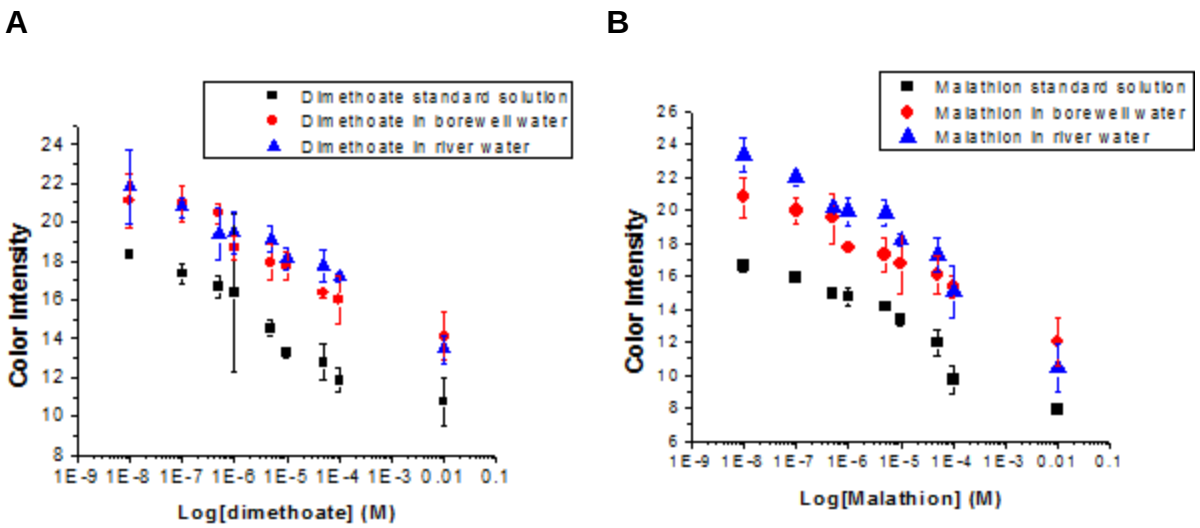


Figure 3.12: comparison of inhibition pattern of commercial pesticides in surface and ground water with standard solution (pesticides in buffered milli-Q water). All points are means ± s.d. of three independent measurements for each concentration.

Figure 3.12 shows a comparison between inhibition plots for standard solutions of malathion, dimethoate and plots for surface water and ground water with commercial pesticides. As shown in figure 3.12, both the surface water and ground water display the trend of the response vs concentration similar to standard solutions of pesticide. Although the inhibition trend has the same pattern, in case of water samples spiked with commercial pesticides, there is difference in color intensity measured compared to standard solutions of the same concentrations. From the data obtained, it can be inferred that based on the measured color intensity, concentration of the pesticide in water sample cannot be determined by comparing with standard calibration curve. The paper-based sensors can be used to detect presence of organophosphorus and carbamate pesticides.

3.8 Assessing sample matrix effects on performance of biosensor:

To further evaluate the potential of paper-based sensor, coconut water samples were spiked with varying concentrations of commercial monocrotophos (10mM – 10nM). pH of coconut water was 6.9. The measurements were

performed by incubating the biosensor strips with coconut water samples containing different concentrations of toxins for 15min and then substrate (IDA) was moved to the sensing zone by dipping substrate region of the biosensor strip in the coconut water sample. The sensors were allowed to dry at room temperature until a stable color was developed. Figure 3.13 shows a comparison between inhibition plots for standard solutions of monocrotophos and for coconut water samples spiked with the toxin. As shown in figure 3.13 coconut water display the similar trend of response vs concentration. The difference in color intensity compared to standard solutions is due to the viscosity of coconut water, which slows down the flow of substrate (IDA) to the sensing zone and thus increases incubation time. Increased incubation time enhances the inhibition of acetylcholinesterase enzyme.

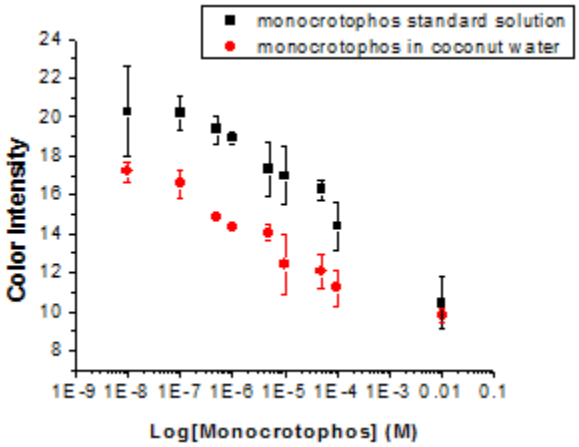


Figure 3.13: comparison of inhibition pattern of commercial pesticides in coconut water with standard solution (pesticides in buffered milli-Q water). All points are means ± s.d. of three independent measurements for each concentration

4 Discussion

4.1 Need for the paper-based sensors:

Pesticides are the one of the toxic compounds consciously released into the environment. Organophosphorus pesticides have been widely used worldwide since the end of the Second World War. More than 100 organophosphorus pesticides are in use and it accounts for the 38% of the pesticide usage worldwide. Even though OP are biodegradable, they are highly toxic to non- target animals, mammals including vertebrates and invertebrates [40]. The world health organization estimates that as many as 3,000,000 people per year are poisoned by pesticides; many are due to OP pesticides, resulting in around 200,000 deaths. The developing world bares the major burden in thisdue to occupational exposure and intentional self- poisoning [41].

As agriculture is one of the main occupations in developing countries, pesticides are used extensively in an unscientific method. Occupational exposure to pesticides is common due to this fact. Death and disabilities due to pesticide exposure is common in many villages of developing country like India, where agriculture is the main occupation in rural areas. These pesticides applied to agricultural land to protect crops finally enter the surface water and ground water through runoff and leaching and contaminate the water. To mention, in one of the recent tragic incident which happened on July 26, 2013, 23 primary school children lost their life by consuming food contaminated with pesticide in Bihar, India was reported in Times of India (http://articles.timesofindia.indiatimes.com/keyword/tragedy/recent/2).

In this study, our data demonstrate the presence of pesticides in three of the sampling points which include both ground water and surface water. Methyl parathion was present in water samples from three different sampling points. According to data collected from local people, these sampling points are the

source for drinking water supply in these villages and no water treatment is done before the water supply except for disinfection. One of the tap water sample (source is ground water) contained four organophosphorus pesticides. Conventional methods for determination of pesticides are time consuming and require sophisticated instruments and trained personnel. The availability of such sophisticated facilities in remote place and less industrialized countries is less probable. All these facts strongly recommend the need of a rapid device, which the common person can use to confirm their drinking water is free from pesticides.

4.2 Importing and testing of strips

The essential criteria required for the storage and shipping of the biosensor strips is the stability of the paper-based sensors. The enzyme and substrate impregnated on the paper should retain its activity in order to be viable. The strips imported for the field study were inactive, pink color developed in the substrate region indicated that the substrate (IDA) on the paper support had oxidized. The silica layers used to immobilize IDA did not help in maintaining the stability of IDA. But silica layers facilitated AChE to be stable on the paper support; activity of enzyme was confirmed by spotting IDA in the sensing region, development of blue color indicated the activity of AChE.

To overcome this issue, we fabricated the paper-based sensors locally by printing enzyme and substrate without the silica and capturing agent. We imported wax outline printed whatman#1 paper sheets. The strips were stored at 4^0C in order to keep the enzyme and substrate viable. The sensor performed similarly but suffered from a drawback of leaching of blue color. This show that, capturing agent is required to hold the color. Our data indicate that, in order to conduct the effective assay with paper-based sensor, further optimization is required to improve the stability of the substrate. Fabrication method of biosensor strips needs further improvement to maintain the consistency of volume of

bioinks printed in order to get the consistent results. The strips need to be optimized to be viable for atleast for a period of 60 days irrespective of fluctuations in temperature in order to facilitate shipping of the paper-based sensors.

4.3 Requirements for useful assay

Paper-based sensors perform in pH range close to pH 8.0. Before conducting the assay pH of the water samples need to be measured. It would be convenient if pH paper is included as part of paper-based biosensor. In case of highly turbid water and samples containing any algae, filtration is required before conducting the assay in order to avoid interference with color developed. A blank assay with ddH_2O is necessary for every test to compare the color intensity developed with sample test. While conducting test with coconut water, pH adjustment is necessary if the pH of coconut water sample is not close to pH 8.0. With the existing fabrication of biosensor strips, the color intensity of the blank strips varies from 28 to 34, these data indicate that there is no consistency in the volume of bioinks printed. Since it is colorimetric assay, it is essential to have consistency of color intensity with blank strips as the color intensity of the sample is compared with the blank. In order to conduct assay with the real world samples, optimization of the fabrication of bioinks to have consistency in volume of bioinks printed is required to get the reliable consistent results. The possibility of printing pH buffers on future version of sensors can be explored, as the measurement is pH sensitive.

5 CONCLUSIONS

In summary, our current study was an attempt to explore efficiency of paper-based biosensors to detect toxins in the samples from different aquatic environment. The design of the paper-based sensor to detect pesticides was modified and bioinks (AChE and IDA) was fabricated on the paper support using inkjet office printer. Assay was conducted using paper-based sensors to determine the pesticides in different water samples including surface water and ground water collected from different villages in southern India. The color intensity is inversely proportional to concentration of toxin. The results obtained from the assay were validated with conventional method. Liquid-liquid extraction combined with GC-MS was used to confirm the results obtained from sensors. The sensor was effective in detecting pesticide in water samples from different sources. The results were in agreement with result obtained from GC-MS. The inhibition trend of response vs concentration of water containing commercial pesticides was similar to standard solutions.

Our experimental data show that the presence of higher concentration of dibutyl phthalate (in the range of 100µM to 10mM) in water can interfere with assay, as dibutyl phthalate inhibit acetylcholinesterase enzyme. The sensor was also successful in determining the toxins in coconut water. The assay showed a negligible matrix effect with pesticide- spiked coconut water, provided the pH of coconut water is in range close to pH 8.0 (pH of the coconut water varies with the age of the coconut water). Our data demonstrate that, AChE and IDA on paper retain its activity for at least 1 month when stored at 4^{o}C, IDA entrapped between two biocompatible silica layers on paper loses its stability with fluctuations in temperature. Thus it requires further optimization to stabilize the IDA, to enable the shipping of the sensors.

On the basis of this study, we come to following conclusions:

- Paper-based biosensor assay platform can detect pesticides in the environmental samples and results have been validated by GC-MS. But for transfer of technology to the industry, further optimization is required to improve the stability of substrate to withstand temperature fluctuation thus allowing the storage and shipment of the biosensor strips. In order to conduct reliable assay and to obtain consistent results, the fabrication of biosensor strips needs to be improved to maintain the consistency with volume of bioinks impregnated on paper support.
- The presence of higher concentration of dibutyl phthalate (in the range of 100µM to 10mM) in water can be potential interference for the paper-based assay for detection of pesticides in water.
- The first generation of the pesticide sensor was similar to that of litmus paper, and it was difficult for common person to identify the sensing and substrate region. The generation 2 sensor has overcome this setback; sensing region is circular in shape and can be easily identified and as the length of the sensor is reduced by half run time is reduced which makes the sensor user friendly.
- Further optimization is required to improve the stability of the substrate (IDA) to make the strips viable. The substrate stability needs to be improved to tolerate the possible temperature fluctuations during the shipment of the paper- based sensors.
- Increasing self-life of the strips to 60days and optimization of biosensor strips to make them temperature insensitive to maintain its stability will facilitate the shipment of the paper-based sensor to remote places for real world application.

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