INTRODUCTION

“New ways to measure toxic substances (chemicals) at trace levels, provide a foundation for more precise assessment of human exposure and risks, and are useful in shaping statutes.”

1.1 PROBLEM STATEMENT, RELEVANCE AND SIGNIFICANCE

“In truth, a big step forward in analytical science, would be an enhancement in sensitively detecting, and reliably quantifying the toxic chemicals in trace concentrations in various water matrices, which stands out as a problem of great scientific, societal and political importance.”

The ability to detect and quantify toxic chemicals with high precision, accuracy and sensitivity, across various samples is an essential task in analytical, biomedical, environmental, regulatory and water research. The analytical protocols are highly desirable, whose work focuses on, new methods in toxic analysis, with applications aimed at improving the quality of human life.

Cataloguing the potential risks that are posed to humans, animals and aquatic life, by toxic substances has resulted in a perceived need for action²⁻⁸, and hence a requirement for ‘usable analytical science’. This is now widely promoted in environmental research programs. The need to assess the human and environmental risks of toxic chemicals have prompted, the development of novel analytical methods using noble and novel analytical tools for their separation, detection, characterization and quantification. Some of these methods have tremendous potential for use in various scenarios of environmental and health sciences. Chemicals are being increasingly incorporated in a wide range of consumer and industrial products⁹⁻¹⁰. Their unique properties determine their fate in the environment, and the nature of their interactions with biological systems. Characterizing samples that have trace concentrations of toxic chemicals, is a challenging task for current analytical science. Existing analytical methods need to be adapted, and new ones to be developed, to generate reliable results for detection, quantification, and risk assessment of toxics in trace concentrations. Given the concerns raised over the potential impact of toxic chemicals on
environmental and human health, the development of a toxic-specific risk assessment strategy is required. The detection and quantification of toxics at trace concentrations requires fit-for-purpose analytical protocols, and techniques that feature high sensitivity, reliability, and accuracy.

There is a growing number of precise, systematic and credible evidences in literature\textsuperscript{11-14}, that many chemicals don't play by the usual rules of toxicology i.e. dose and effect move together in a predictably linear fashion and chemicals toxicity at low doses is not predicted by effects at higher doses. The problem with the trace amounts of toxics is that they may appear harmless in a quick test for toxicity but as, “absence of evidence is not the evidence of absence”, with continuous exposure at trace level, after few days it may have a big effect. Clearly, enhanced sensitivity is an ongoing need that demands continuing innovation – especially, to gain the robustness.

Every now and then analytical limitations i.e. limit of quantification/limit of detection (LOQ/LOD) are limiting factors for deciding this health-based tolerable or allowed maximum concentration limit (MCL). The analytical protocols must have existed prior to the reporting of the tolerable or allowed MCL, with appropriate LOQ values, and acceptance of these limits as valid, implies that the analytical protocols used, are regarded as satisfactory, and that they have been applied in a proper manner. On the other hand, an information on the magnitude window of the MCL is essential to the analyst in the selection of suitable protocols/methods for the analysis. When a method of this type is requested, the most important question, is "what is the order of concentration (milligram, microgram, picogram or less) to be measured, and with what precision (15%, 20% or other value), is to be measured?"

Thus, tolerable or allowed maximum concentration limit (MCL) value influences the design of an analytical protocol, but, in at least one respect, the attitude of the analyst i.e. LOQ values may exert some pressure on the approach of the regulatory agencies towards these limits.

The lower analytical limit (LOQ/LOD) of an analytical protocol is controlled by the sensitivity of the instrument, by the volume of sample taken, and by the magnitude of the blank value which in turn is a matter of the quality of the reagents. By using a sufficiently insensitive method, and sufficiently impure reagents, an analyst can make almost any toxic concentration show no significant difference from the blank, which is
the only meaningful definition of a zero concentration (ideal value). Conversely, if the analyst is given a definite concentration range or lowest possible practical value to aim at, even though, it be as low as the minute value of nanogram or picogram, by the development of a sensitive protocol, can produce an answer with the agreed precision.

Daily routines expose many people to potentially toxic substances, including bisphenol A, di-2-ethylhexyl phthalate, pharmaceuticals, polycyclic aromatic hydrocarbons and nitrophenols, few to be mentioned here. Estimates for the predominant toxic compounds or direct measurement in various environmental compartments in one’s day illustrate how much one’s exposure can fluctuate, with the highest levels of exposure may typically arising during indoor activities. Most environmental laws seek to control only the release of potentially toxic substances into the air and water, soil, not the amount of contact, people actually have with those toxic substances.

*The focus on emissions rather than exposure essentially disregards the reality that toxic substances produce health risk only if they reach the human body.* Toxic substances exposure is through different media i.e. food, water, air and various consumer products. Now to explicate human exposure to the chemicals of concern stemming from sources of these chemicals occurring within both the “nearfield” and “farfield”. Farfield sources are defined as relatively large but initiated as typically distant sources or emissions to the general environment (air, water, soil). “*Near field sources are those that occur within the ambience of a residence or literally at arm’s length for the exposed person. The near field has been shown to be the dominant milieu for human exposure for many toxic chemicals, if not the majority of chemicals, especially those that are not present at relatively high levels in the general environment i.e. present at trace levels*”. On that pitch water is on the top of the list in terms of nearfield exposure medium, after air i.e. next to air, water is the most accessed medium of exposure in various forms of daily routine. Thus, a critical challenge for any monitoring analytical design to measure these chemicals at trace concentrations and consequently to really understand human (especially consumer) exposure to these trace chemicals, and its potential effects on human health, lies in the nearfield water zone i.e. drinking and source waters.

*The time is running out and the scientific, economic, political, and social challenges and overlapping needs are enormous, desires for the innovation of novel,
noble, precise, accurate and sensitive analytical protocols for the analysis of low-concentration/trace, high impact toxic substances in water, to maintain water quality in the face of modern-day needs, and consequently for a better, close and desirable coordination with toxicologists, to assess the toxicity more precisely, and to derive striking policy decisions.

1.2 RESEARCH OBJECTIVES

The "chemical universe", the diversity of combinations and forms in which chemical compounds occur, is immense. It is highly challenging to track these processes and to identify the toxic substances, particularly at low concentration levels. This is the area on which this Ph.D. thesis research work in analytical chemistry, focuses. In terms of analysis, we aim to develop very effective and sensitive methods to quantify a broad spectrum of toxic substances at trace and ultra-trace levels.

To achieve this aim, this research work has the following subordinate objectives:

1. Separation and identification of known/emerging targeted toxic chemicals in drinking and environmental waters.
2. To innovate novel methods that focus on increased sensitivity.
3. To innovate green analytical protocols with complete validation.
4. Determination of trace and ultra-trace concentrations of toxics.
5. Analysis in shorter time.
6. Analysis from smaller sample volumes.
7. Cost-effective analysis.
8. Application of developed protocols to real world water samples.

Our goal is to develop, and to make use of innovative analytical protocols/methods to determine trace and ultra-trace concentrations of toxic substances in the water matrices. This work could support, and play a pivotal role in the assessment of these toxic chemicals and the understanding of translocation, transformation and removal processes.

1.3 TOXIC SUBSTANCES

1.3.1 Toxic tale

There is a plenty of research work on toxic chemicals, solely about the well-known legacy toxics, polychlorinated biphenyls (PCBs), polybrominated biphenyls
(PBBs), dichlorodiphenyltrichloroethane (DDT), metals and dioxins. Every week, there is an abundance of news, articles revealing the widespread contamination of drinking water, environment, wildlife with so-called emerging contaminants such as phthalates (used to plasticize polyvinyl chloride), bisphenol A (used in the production of polycarbonate plastic), pharmaceuticals, nitrophenols, polyaromatic hydrocarbons.

An emerging contaminant can be a newly recognized entity or a known compound that presents new characteristics or risks. Although most of these so-called emerging contaminants have been around for decades, they have only recently “emerged” into our collective consciousness. In either case, new analytical approaches may be required to guarantee consumer safety.

There are several reasons that we are now hearing more about them: Thanks to much-improved detection tools and technologies, chemists can now find these substances in water, food, and biological samples, in concentrations as minute as a few parts per trillion or even below. In addition, over the past couple of decades, as better methods of testing have allowed the evaluation of smaller (and often, more environmentally relevant) concentrations, toxicologists have expanded the definition of “adverse effect” to include the subtle effects that tiny amounts of toxic chemicals may have on reproductive and developmental processes in people and animals.

Terminology and definitions for substances/chemicals that cause toxic effects are not always consistently used in the literature. The most common terms are toxicant, toxic, toxin, poison, toxic agent, toxic substance, and/or toxic chemical.

The term "toxic" refers to a chemical substance that is harmful to biological systems. In the course of quotidian activities, people are spasmodically exposed to a variety of toxic chemicals, through various media and pathways of exposure, including air (inhalation), water and food (ingestion) and surfaces (dermal absorption). The majority of toxic substances in ambient air originate from sources that emit to the outdoors, such as vehicle emissions, drycleaners and power plants. However it is clouded that only whether these sources are the predominant contributors to human exposure. Although not major sources of emissions, personal activities and indoor sources may also be the dominant sources of exposure for many toxic compounds. Some examples of sources of personal exposure to toxic compounds include household
cleaners, drycleaned clothes, vehicle exhaust, gasoline vapors and environmental tobacco smoke (ETS)\textsuperscript{23-27}. Food items, both solid and liquid food items, may become contaminated through contact with chemical agents during growing, harvesting, processing, packaging, transportation, and distribution\textsuperscript{28-31}. Chemical residues in water, include synthetic chemicals (plasticizers), insecticide and herbicides, domestic and industrial waste, pharmaceuticals, toxic metals and organic compounds\textsuperscript{32-36}.

Toxic substances vary widely in the types of harm they cause and the conditions under which they become harmful. The effects of the toxic substances vary widely, too. Acute reactions are sudden ones such as vomiting or dizziness. Chronic reactions occur over longer periods and include symptoms such as decline in mental alertness, change in behavior, cancer, and mutations that can harm unborn children of exposed parents. Because toxics can cause both acute and chronic reactions, they are a broader category than poisons, which produce acute reactions only. For this reason, the words toxic and poison are not interchangeable\textsuperscript{37}.

Chemicals used in general, that is, in the industry, in commercial and consumer products, agricultural products, have always presented a number of challenges for regulators. First, there are a large number of chemicals in use in such products. For example, European Inventory of Existing Commercial Chemical Substances lists 100,000 chemicals\textsuperscript{38} whereas the US inventory of existing chemicals under the Toxic Substances Control Act (TSCA) is approximately 70,000\textsuperscript{39-40}. Second, there are a wide range of chemicals used in various products. Third, one chemical may result in a number of different exposures to different individuals by different routes. Finally, the nature of the chemical exposures that result from the use of these products (chemicals within) is highly variable. The amount of the exposures typically varies.

On exposure platform, water contamination/pollution is a serious issue because it affects our lives and is expected to get worse over coming decades\textsuperscript{41-43}. Water pollution is any contamination of water with chemicals or other foreign substances that are detrimental to human, plant, or animal health. These pollutants include fertilizers and pesticides from agricultural runoff; sewage and food processing waste; lead, mercury, and other heavy metals; chemical wastes from industrial discharges; and chemical contamination from hazardous waste sites. Worldwide, nearly 2 billion people
drink contaminated water that could be harmful to their health. There is increasing evidence that the use of chemicals frequently results in widespread water contamination/pollution with little understanding of the toxicological implications. More than seven hundred organic and inorganic chemicals have been reported in water. Among these, certain organic and inorganic chemicals are dangerous because of their highly toxic and carcinogenic nature and may have long persistence in the environment. Much attention has focused on compounds known to exhibit toxic activity at low concentrations and most toxic organic pollutants/contaminants are pesticides, polynuclear aromatic hydrocarbons (PAHs), plasticizers, drug residues, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), phenols and toxic metal ions.

Over half of the world’s hospitals beds are occupied with people suffering from illnesses linked with contaminated water and more people die as a result of polluted water than are killed by all forms of violence including wars.

The primary anthropogenic sources of water contamination are human and animal waste, domestic, industrial and agricultural runoffs. Contamination occurs when agents of water-related illness and nutrients, such as nitrogen and phosphorus, are carried from urban, residential, and agricultural areas into surface waters, groundwater, and coastal waters. The nutrient loading can promote growth of naturally occurring pathogens and algae. Figure 1 schematically demonstrates the toxic tale of water contamination. Human exposure to water contaminants occurs via several pathways including, drinking water sources including bottled water also, daily use of water for different purposes i.e. washing, bath, sanitation, recreational waters, fish and shellfish and have adverse affect on human health.

There is still time to tackle water contamination. Better water monitoring protocols and methods, are needed to understand the scale of the analytical and purification challenges around the world and to identify key solutions. Once in-depth assessments have been done there are a raft of new analytical methods that can help to locate and reduce the contamination at source, treat polluted water before it enters water bodies, and protect human health and ecosystem.
“There is no doubt that we have analytical tools and techniques, to innovate remarkable analytical methods needed to tackle this growing toxic substances problem in the environment, it is now time to stretch, push and use these analytical tools, to combat the toxics problem at various levels, which at present, is one of the greatest threats to human health and environment around the world.

<table>
<thead>
<tr>
<th>Human and animal waste</th>
<th>Industrial outfalls</th>
<th>Agricultural runoffs</th>
<th>Domestic outfalls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxic algal blooms</td>
<td>Water contamination</td>
<td>Plastic products/waste</td>
<td>Flushed drugs</td>
</tr>
<tr>
<td>Pathogens</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TOXICITY

Decreased human health

Figure 1.1 | Schematic diagram for toxic tale of water contamination.

1.3.2 Flagged chemicals

A total of 12 different chemicals have been flagged in the context of this Ph.D. thesis, include Bisphenol A (BPA); three isomeric mononitrophenols (MNPs) (ortho, meta, para); three estrogens namely, 17α-ethinylestradiol (EE2), 17β-estradiol (E2), and diethylstilbestrol (DES); four polyaromatic hydrocarbons (PAHs) namely, anthrancne (Anth), fluoranthene (Flu), pyrene (Pyr), and phenanthrene (Phen), and a phthalate namely, di-(2-ethylhexyl) phthalate (DEHP). The estrogens, MNPs and PAHs are clearly multi-component in nature, whereas BPA and DEHP are generally considered single compounds for regulatory purposes. Choices of target toxics are based on the criteria such as prevalence in drinking and environmental water, toxicological significance, human exposure, scientific, societal and political concern and, pitfalls and peculiarities of the reported literature along with the practical bench criteria such as availability of economic analytical standards, chemical reagents, and analytical techniques were deliberately considered. In the following paragraphs, these targeted toxics would be discussed with a attention on their properties, human exposure, toxicological profile and guidelines and regulations.
1.3.3 BISPHENOL A

*Identified as endocrine disruptor, potential carcinogen,*
*Social, environmental, and a global controversial issue,*
*Also known as BPA, 2, 2-bis-(4 hydroxyphenyl) propane or 4,4’-isopropylidenediphenol.*

Bisphenol A is one of the world's highest production volume chemicals, with more than 2 million metric tons produced worldwide in 2003 and increase in demand of 6% to 10% annually. Bisphenol A was first synthesized by the Dianin in 1891. The compound is synthesized by the condensation of acetone (hence the suffix A in the name) with two equivalents of phenol. The reaction is catalyzed by a strong acid, such as hydrochloric acid (HCl) and industrially, a large excess of phenol is used to ensure full condensation, reaction scheme shown as follows:

![Chemical Reaction Diagram](image)

Phenol + Acetone + Phenol → BPA

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1.3.3.1 Properties, uses and occurrence

Bisphenol A is an organic synthetic chemical, is a white solid powder at room temperature with a mild phenolic odour. It is practically soluble in water, and easily soluble in aqueous alkaline solution, alcohol, and acetone. Physical and chemical properties of BPA are listed in the Table 1.1.

BPA, is widely used as a monomer, to make polycarbonate plastics and epoxy resins, which in turn find application in a wide variety of domestic products. BPA is present in dental fillings, plastic food and water containers, water bottles, bottle tops, water supply pipes, baby bottles, food wrap, medical devices as well as in the lining of beverage and food cans, presenting a large number of routes for, widespread, and continuous, human exposure to BPA.

Unfortunately, the chemical bonds that link BPA in polymer structures are not completely stable and the polymer may slowly decay with time, especially at high
temperatures releasing small amounts of BPA, into materials with which it comes into contact, such as food or water.

Table 1.1 | Physicochemical properties of BPA.*

<table>
<thead>
<tr>
<th>Property</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C₁₅H₁₆O₂</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>228.287</td>
</tr>
<tr>
<td>Colour</td>
<td>white</td>
</tr>
<tr>
<td>Odour</td>
<td>Mild phenolic</td>
</tr>
<tr>
<td>Melting point</td>
<td>153 °C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>360.5 °C at 760 mm Hg</td>
</tr>
<tr>
<td>pKₐ</td>
<td>9.6</td>
</tr>
<tr>
<td>Solubility</td>
<td>In water, 300 mg/L at 25 °C, soluble in aqueous alkaline solution, alcohol, acetone, acetic acid</td>
</tr>
<tr>
<td>Log Kₐow</td>
<td>3.32</td>
</tr>
<tr>
<td>Chemical structure</td>
<td></td>
</tr>
</tbody>
</table>

*Source: HSDB (Hazardous Substances Data Bank, U.S.)

Although hydrolysis of the ester bond linking BPA molecules in plastic, results in leaching of BPA in water/food, is facilitated by heating, normal use such as storage, can also result in polymer degradation leading to release of BPA. Basic and acidic beverages and foods, as well as fatty foods, increase the rate of leaching of BPA. Numerous studies have confirmed leaching of BPA from packaged food, and detectable levels of BPA are present in a wide range of food containers.

1.3.3.2 Exposure and toxicological profile

Evidence exists that urinary BPA levels are positively correlated with the consumption of canned foods, suggesting that oral exposure is likely the primary source of human exposure to BPA. Small quantities of BPA have been detected in sediments and river water and, in domestic dust and air samples. BPA also accounts for the estrogenic activity that leaches from landfills into the surrounding, treatment of leachate, effluent from industrial activity, may serve as an route of human
exposure, particularly if it finds its way into aquatic environment. Given the ubiquity of BPA in environment, it is not surprising that exposure to BPA is virtually universal. Data also confirm the passage of BPA across the placenta. Breast milk is an additional route of exposure of BPA to offspring. A study reported BPA in human breast milk samples, up to 6.3 ng/mL and concentrations of 1 to 7 ng/mL in the mother’s colostrum tested. BPA in drinking water and its source waters have been reported in numerous studies and represent drinking water as a relevant source of human exposure and risk.

The National Institute of Environmental Health Sciences (NIEHS) U.S. invested approximately $30 million on BPA research.

Scientific dogma holds that BPA is metabolized and it is excreted quickly. However, research have found that parent BPA in blood of pregnant women and in newborns, suggesting either continuous and widespread exposure to BPA, or alternatively, that only a portion of BPA is metabolized and excreted. Urinary BPA levels have not been shown to decline consistently with increasing fasting times; this finding suggests that dietary exposure may not be the only important source of exposure, or that BPA is not cleared rapidly from the body. The low-dose exposures to BPA (> 200 times below the U.S. EPA recommended dose) in pregnant mice negatively affected mammary gland development of female offspring by increasing ductal area and extension, promoting fat pad maturation, and decreasing epithelial cell size. In addition, tumor-promoting agonistic effects of BPA on a mutant androgen receptor in a human prostate adenocarcinoma cell line were more pronounced at low doses than at higher doses. Thus, a need exists to better understand how chronic exposure to low doses of BPA may affect human health.

Among the many health effects associated with BPA exposure, the chemical has been linked with abnormal male and female reproductive organ development in animals and sperm anomalies in humans. There is some concern for neural and behavioral effects in fetuses, infants and children at current human exposures.

Reproductive tract anomalies following prenatal or neonatal BPA exposure provide another potential means to adverse perinatal outcomes. Several recent studies have reported that fetal and neonatal exposure to BPA may impact fertility, age of
reproductive senescence and onset of disease later in life. BPA exposure has been found to be associated with timing of puberty. Human studies have shown that BPA is elevated among women with ovarian dysfunction, including women with polycystic ovaries and endometrial hyperplasia. Support for increased risk of BPA exposure with obesity comes from studies that document higher levels of BPA in obese women. BPA also accumulates in body fat, which in turn could be mobilized during pregnancy and lactation.

Academic researchers who have studied a wider range of BPA doses, including very low ones found in the everyday environment, say that their experiments usually do not generate the tidy, familiar 'ski-slope' dose-response graphs of classic toxicology. Instead, BPA has 'non-monotonic' dose-response curves, meaning that their slopes change at least once from negative to positive, or vice versa, forming 'U' shapes, inverted 'U's or even stranger shape. BPA has a non-monotonic dose–response curve and, as a result, there is controversy regarding the level of a “safe” dose.

Various associated health effects of BPA has been summarized briefly as follows:

- Bisphenol A has been linked to various health issues and risks
  - Changes in brain and behaviour changes
  - Prostate cancer and breast cancer
  - Early onset to puberty
  - Miscarriage and birth defects
  - Effects on fertility
  - Diabetes and obesity

1.3.3 Guideline

According to U.S. EPA guidelines, safe levels of BPA intake are 50 μg/kg body weight/day, assuming that the main source of exposure is from ingestion. Nevertheless, studies suggest that the U.S. EPA guidelines and the conclusion that current dietary intake levels are safe should be reevaluated.

1.3.4 MONONITROPHENOLS

Three isomeric form with molecular formula: C₆H₅NO₃

- 2-nitrophenol, 3-nitrophenol, 4-nitrophenol

Hazardous waste, Priority pollutants.
There are three isomeric forms of mononitrophenol (MNP), namely 2-nitrophenol (2-NP), 3-nitrophenol (3-NP), and 4-nitrophenol (4-NP). These three MNPs are widely applied as intermediates in the production of pesticides, fine chemicals and pharmaceuticals. During the application process of these compounds, they are inadvertently released into the environment to contaminate rivers and ground waters. Thus, it is easy for them to enter into the environmental water samples, and then cause significant damages to the human health due to their high toxicity and low degradability in the ecological environment. They are listed as the priority toxic pollutants, due to their toxic effect on humans, animals and even at low concentration. The toxicity of three isomers is different from each other, 2-NP and 3-NP present more adverse effects on the development and metabolism of organism and 4-NP the most toxic. They have great potential toxicities of carcinogenesis, teratogenesis, and mutagenesis. There are no known natural sources of these nitrophenol isomers. Environmental releases to air, water, and soil are from various anthropogenic sources, i.e. vehicle traffic, industrial emissions, and degradation of the pesticides. Three isomers, has been discussed in paragraphs below, with their physico-chemical properties summarized in Table 1.2.

Information found on 3-nitrophenol was very scarce, and this isomer is much less prevalent in industry and in the environment, thus concluding only a brief discussion in the last and 2 and 4-nitrophenol have been discussed together in the following paragraphs.

1.3.4.1 2-nitrophenol and 4 nitrophenol
Also known as ortho-nitrophenol, o-hydroxynitrobenzene, 1-hydroxynitrobenzene and para-nitrophenol, p-hydroxynitrobenzene, 3-hydroxynitrobenzene, PNP respectively.

2-Nitrophenol and 4-Nitrophenol are mainly used to produce dyes, paint coloring, rubber chemicals, substances that kill molds (fungicides), insecticides (parathion), drugs (acetaminophen) and to darken leather and consequently nitrophenolic effluents are relatively common in industrial wastes. 2-NP and 4-NP have been detected in the exhaust gases of diesel vehicles and light-duty gasoline. 2- and 4-nitrophenol are also generated in the atmosphere during the photochemical degradation of aromatic compounds such as benzene and toluene in the presence of hydroxyl radicals or nitric oxide and nitrous dioxide.
Table 1.2 | Physicochemical properties of MNPs.*

<table>
<thead>
<tr>
<th>Property</th>
<th>2-nitrophenol (o-nitrophenol)</th>
<th>3-nitrophenol (m-nitrophenol)</th>
<th>4-nitrophenol (p-nitrophenol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C₆H₅NO₃</td>
<td>C₆H₅NO₃</td>
<td>C₆H₅NO₃</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>139.10</td>
<td>139.10</td>
<td>139.10</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>44-45</td>
<td>96.8</td>
<td>113-114</td>
</tr>
<tr>
<td>Boiling point (°C)</td>
<td>216</td>
<td>194</td>
<td>279</td>
</tr>
<tr>
<td>log Kow</td>
<td>1.79</td>
<td>2.0</td>
<td>1.91</td>
</tr>
<tr>
<td>pKa (25°C)</td>
<td>7.23</td>
<td>8.36</td>
<td>7.15</td>
</tr>
</tbody>
</table>

Molecular structure

*Source: HSDB (Hazardous Substances Data Bank, U.S.)

Significant releases of 4-NP into the environment may occur from the hydrolytic degradation of the insecticides parathion and parathion-methyl and from the photolytic degradation of the herbicide nitrofen.¹⁰⁷

Humans may be exposed to 2- and 4-NPs via inhalation and skin contact during production and processing (mainly in the manufacturing of pesticides). An exposure of the general population to nitrophenols via the environment, predominantly through ambient air, drinking and source waters.

1.3.4.2 Toxicological profile

There is only limited information concerning the toxicological profiles of 2- and 4-NP. 2-NP is slightly irritating to the skin but non-irritating to the eye. NPs can be classified as substances exhibiting moderate to high toxicity in the aquatic compartment. 4-NP was found to be more toxic than 2-NP. A dose-dependent increase in the formation of methaemoglobin was seen in cats after oral exposure to 2-NP and in rats after exposure by inhalation to 4-NP. After repeated exposure to 4-NP, the formation of methaemoglobin was shown to be the most critical end-point for exposure.
by inhalation and is assumed to be relevant for oral exposure too. Other noted effects included decreases in body weight gain, differences in organ weights, focal fatty degeneration of the liver, and haematological changes. For these effects it was not possible to identify a clear dose–response relation or reliable no observed adverse effect Levels.98-100.

Considerable work is needed to better understand three mononitrophenols toxicity, transformation, mechanisms of action, and exposure levels to ensure that nitrophenols uses and exposures are consistent with basic safety standard, namely that there is a ‘reasonable certainty of no harm’ from ongoing, acute and chronic exposures to nitrophenols across the world population.

No sufficient data on 2-NP, to allow any conclusions to be made about its possible mutagenicity. 4-NP can cause chromosomal aberrations in vitro. From valid test results available on the toxicity of 2- and 4-NP to various aquatic organisms, NPs can be classified as substances exhibiting moderate to high toxicity in the aquatic compartment. Therefore, despite biotic and photochemical decomposition, NPs emitted to water could pose some risk to sensitive aquatic organisms, particularly under surface water conditions not favouring both elimination pathways. Because of their use patterns and release scenarios, it is likely that NPs pose some risk to aquatic organisms.100

1.3.4.3 3-nitrophenol

Also known as meta-nitrophenol, meta-hydroxynitrobenzene, 2-hydroxynitrobenzene.

3-NPs production and use as an intermediate in synthesizing dyestuffs and drugs and its use as an indicator may result in its release to the environment through various waste streams. If released to air, 3-NP will exist solely as a vapor in the ambient atmosphere. 3-NP absorbs strongly above 290 nm (UV light)) which results the direct photolysis of 3-NP in the environment. As in table 1.2, the pKa value of 3-NP is 8.36, indicated that this compound will partially exist in anion form in the environment and do not adsorb more strongly to soils containing organic carbon. If released into water, 3-NP may have little to moderate adsorption to suspended solids and sediment in the water and was readily biodegradable in river water die-way tests, suggesting that biodegradation may be an important environmental process in water. Exposure to 3-NP may occur through occupational dermal contact at workplaces.
where 3-NP is produced or used. Effects from exposure may include burns to the skin and eyes, headache, dizziness, shock, unconsciousness, and death from cardiac or pulmonary failure.  

1.3.4.4 Guidelines and regulations  

There is no criteria for MNPs in U.S. EPA guidelines, to protect human health from adverse effects of MNPs ingested in contaminated water and fish. Although there are recommendations, to protect freshwater aquatic life and are as follows:  

2-NP -- 2700 µg/L and should not exceed 6200 µg/L.  

4-NP -- 240 µg/L and should not exceed 550 µg/L.  

For NPs, the US EPA recommends restricting the concentrations in natural water bodies to below 10 ng/L.  

1.3.5 ESTROGENS  

17α-ethinylestradiol (EE2), 17β-estradiol (E2), and diethylstilbestrol (DES),  

Constituents of pharmaceuticals, birth-control pills, estrogen replacement therapies,  

Human carcinogens, presence in water systems is a major environmental issue.  

Trace concentrations of endocrine-disrupting chemicals (EDCs) in the environment, including synthetic and natural hormones, have caused adverse impacts on aquatic organisms. Possible sources of these chemicals to the environment include discharges from wastewater treatment plants, domestic septic systems, use of reclaimed water for irrigation, effluents from animal feeding, and runoff from agricultural fields where manure and biosolids – organic-rich solids resulting from treatment of sewage sludge – are applied as fertilizers and soil amendments.  

Estrogens are the primary sex hormones in females. Estrogens are found in both, males and females. In males, estrogen is important in the maturation of sperm. In females, estrogen is important in the regulation of the menstrual cycle, in the development of secondary sexual characteristics, and in pregnancy. Estrogen also plays an important role in normal bone development and maintenance in both males and females.  

*Estrogen may also be refer to, any substance, natural or synthetic, that mimics the effects of the natural hormone and may impact a broad range of health effects at very low levels — well below the “no effect” levels determined by traditional testing.*
17α-ethinylestradiol (EE2) and 17β-estradiol (E2) are steroidal estrogens, which are structurally related hormone molecules derived from the cholesterol molecule. The steroid 17β-estradiol is the most potent and prevalent endogenous estrogen. It is the predominant estrogen in non-pregnant women, and is secreted by the ovaries in women with normal menstrual cycles, and by the placenta in pregnant women. Steroidal estrogens are fat-soluble (lipophilic) molecules that are essential for the growth, differentiation, and function of tissues in humans and other vertebrate animals. In the brain, estrogen affects factors, regulating procreation including mood, reproductive behavior, and production and release of gonadotropins from the pituitary.

E2 occurs as an odorless, white crystalline powder with a molecular weight of 272.38 and a melting point of 178.5 °C. EE2 occurs as an odorless, yellowish crystalline powder with a molecular weight of 296.4 and a melting point of 182°C to 184°C for the more stable form and 142°C to 146°C for the less stable form. Physical and chemical properties of both EE2 and E2, are listed in the following Table 1.3.

Diethylstilbestrol (DES) is a synthetic non-steroidal estrogen that is an odorless white crystalline powder at room temperature. Diethylstilbestrol (DES) was first synthesized in 1938 and was the first synthetic estrogen. It is practically insoluble in water and soluble in methanol, ether, chloroform, acetone, hydroxides, dioxane, ethyl acetate, methanol. The trans-isomer is used for commercial purposes and is stable in the environment. The cis-isomer is not stable and tends to convert to the trans form. Physical and chemical properties of diethylstilbestrol are listed in the following Table 1.3.

1.3.5.1 Use

Both naturally occurring estrogen (E2), and synthetic estrogen (EE2) are widely used medicinal drugs. E2 is essential for normal maintenance and growth of the lining of the uterus, for development of secondary and accessory female sex characteristics, and for pregnancy. Since 1960, estrogens have been used in oral contraceptives. Steroidal estrogens, most commonly EE2, are also used with various progestogens in combined oral contraceptive formulations. Currently, many of the oral contraceptives contain either 30/35 μg of EE2, because this dose has contraceptive efficacy, is well tolerated, and has a low risk of side effects e.g. breakthrough bleeding. EE2 and E2 are used in a variety of veterinary treatments and also in biochemical research.
### Table 1.3 | Physicochemical properties of targeted estrogens.

<table>
<thead>
<tr>
<th>Property</th>
<th>17α-ethinylestradiol (EE2),</th>
<th>17β-estradiol (E2),</th>
<th>Diethylstilbestrol (DES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{20}H_{24}O_{2}</td>
<td>C_{18}H_{24}O_{2}</td>
<td>C_{18}H_{20}O_{2}</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>296.40</td>
<td>272.38</td>
<td>268.36</td>
</tr>
<tr>
<td>Form/colour</td>
<td>Crystalline white powder</td>
<td>White crystalline powder</td>
<td>White crystalline powder</td>
</tr>
<tr>
<td>Odour</td>
<td>Odourless</td>
<td>Odourless</td>
<td>Odourless</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>142-146</td>
<td>178.5</td>
<td>169-172 deg C</td>
</tr>
<tr>
<td>Water Solubility</td>
<td>11.3 mg/L</td>
<td>3.90 mg/L at (27 °C)</td>
<td>12 mg/L</td>
</tr>
<tr>
<td>log K_{ow}</td>
<td>3.67</td>
<td>4.01</td>
<td>5.07</td>
</tr>
<tr>
<td>Boiling Point</td>
<td>N.A.*</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Chemical structure

![Chemical structure of estradiol](source)

![Chemical structure of diethylstilbestrol](source)

*Source: HSDB (Hazardous Substances Data Bank, U.S.)*

DES was widely used in U.S., primarily as a treatment to prevent premature deliveries and miscarriages. In 1971, DES was linked to a rare vaginal cancer (clear-cell adenocarcinoma)\(^{105}\) and consequently United States Food and Drug Administration (U.S. FDA) advised physicians to stop prescribing diethylstilbestrol. Other uses included, relief or prevention of postpartum breast engorgement, control of menstrual disorders, hormone-replacement therapy, palliative therapy for cancer of the prostate in men and breast cancer in postmenopausal women, and as contraceptive. Diethylstilbestrol sometimes was given in combination with vitamins, androgens, and antibiotics. Diethylstilbestrol has been used in veterinary medicine and as a growth promoter (as a feed supplement) in sheep, cattle, and poultry\(^{106}\). Its use as a growth promoter was banned in 1979\(^{107}\). It has also been used in clinical trials for treatment of prostate and breast cancer and in biochemical research\(^{108-109}\).

### 1.3.5.2 Exposure

Under normal conditions, the ovaries produce estrogens in response to pituitary hormones. Estradiol is the main naturally occurring estrogen. Meat and milk also may contain estrogens\(^{110}\). Veterinary use of steroidal estrogens (to promote growth and treat
illnesses) can increase estrogens in tissues of food-producing animals to above their normal levels. Conjugated estrogens used in combined oral contraceptives are available as tablets, and those used for postmenopausal estrogen therapy are also available in tablets, transdermal patches and gels, vaginal inserts and creams, subcutaneous implants, and injectable formulations. In 2009, over 84 million prescriptions were filled for brand-name and generic products containing estrogens as an active ingredient\textsuperscript{111}. Potential exposure to steroidal estrogens in the workplace may occur through inhalation or dermal contact during production, processing, or packaging.

Most current exposure to diethylstilbestrol is through its oral administration as a drug in clinical trials for the treatment of prostate and breast cancer. Exposure also occurred in the past through the use of diethylstilbestrol to prevent miscarriages, as hormone replacement therapy, to treat prostate cancer, and in other medical therapies. Many different forms of diethylstilbestrol, including oral tablets (0.1, 0.25, 0.5, 1, and 5 mg), injectable solutions (0.2, 0.5, 1, and 5 mg/mL), and a vaginal suppository (0.1 and 0.5 mg) were approved by the FDA in 2009. Diethylstilbestrol diphosphate also was available as oral tablets (50 mg) and as an injectable solution (250 mg/50 mL). Diethylstilbestrol residues were detected in beef and sheep livers in 1972 and 1973. When diethylstilbestrol was used as a growth promoter for sheep and cattle, people could have been exposed to it at concentrations of up to 10 ppb in beef and mutton.

The concentration of diethylstilbestrol in ambient-air samples from plants that manufactured diethylstilbestrol ranged from 0.02 to 24 μg/m\textsuperscript{3} \textsuperscript{112}. The National Occupational Exposure Survey estimated that 1,492 workers, including 934 women, potentially were exposed to diethylstilbestrol during its manufacture or during product formulation\textsuperscript{113}.

1.3.5.3 Toxicological Profile

Steroidal estrogens are known to be human carcinogens based on sufficient evidence of carcinogenicity in humans. Human epidemiological studies have shown that the use of estrogen replacement therapy by postmenopausal women is associated with a consistent increase in the risk of uterine endometrial cancer and a less consistent increase in the risk of breast cancer. Some evidence suggests that oral contraceptive use also may increase the risk of breast cancer. The increased risk of endometrial cancer is associated with increasing duration of estrogen therapy used to relieve symptoms of menopause, as well as a small increased risk of breast cancer\textsuperscript{114-118}. The estrogen
therapy is also associated with ovarian cancer\textsuperscript{119}. In 2009, IARC concluded there was sufficient evidence of the carcinogenicity of estrogen-only therapy in humans based on increased risks of endometrial cancer and ovarian cancer and limited evidence based on increased risk of breast cancer\textsuperscript{120-123}.

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**Toxic link of estrogens to humans is the various types of associated cancers**\textsuperscript{114-124}.

DES causes a clear-cell adenocarcinoma, a rare cancer of the vagina and cervix. This type of cancer, which typically develops in elderly women, developed in diethylstilbestrol daughters between the ages of 10 and 30 years. Most (though not all) case-control studies found that \textit{in utero} exposure to diethylstilbestrol also increased the risk of testicular cancer in males i.e. diethylstilbestrol sons. The women who took DES at high doses during pregnancy were at increased risk for breast cancer and cancer may have a long latency period (15 to 20 years), but the evidence is inconclusive. As has been found for other estrogens, DES also increases the risk of endometrial cancer\textsuperscript{124-126}.

**1.3.5.4 Guidelines and regulations**

The reported predicted-no-effect-concentrations (PNECs) for protecting fresh water biota are 0.5-0.75 ng/L for EE2, 1 ng/L for E2\textsuperscript{127}. E2 and EE2 are in the contaminant list 3 established by the United States Environmental Protection Agency (U.S. EPA), in which several compounds, still not regulated, are selected to be controlled since they are considered as potential drinking water pollutants\textsuperscript{128}.DES: EPA Reportable quantity (RQ) = 1 lb i.e. 0.454 kg.

**1.3.6 POLYCYCLIC AROMATIC HYDROCARBONS**

\textit{Reasonably anticipated to be human carcinogens,}

\textit{Also known as PAHs or polynuclear aromatic hydrocarbons.}

The term \textit{“polycyclic aromatic hydrocarbon”} (PAHs) commonly refers to a large class of organic compounds that contain carbon and hydrogen and consist of two or more fused aromatic rings. PAHs containing up to six fused aromatic rings are often known as “small” PAHs, and those containing more than six aromatic rings are called “large” PAHs. The majority of research on PAHs has been conducted on small PAHs due to the availability of samples of various small PAHs. The simplest PAHs, as defined by the International Agency for Research on Cancer, are phenanthrene and anthracene, which both contain three fused aromatic rings\textsuperscript{129}. 

---
The general characteristics of PAHs are high melting and boiling points (therefore they are solid), low vapor pressure, and very low aqueous solubility. The latter two characteristics tend to decrease with increasing molecular weight, on the contrary, resistance to oxidation and reduction increases. Aqueous solubility of PAHs decreases for each additional ring. Meanwhile, PAHs are very soluble in organic solvents because they are highly lipophilic. With each additional ring, aqueous solubility of PAHs decreases. PAHs also manifest various functions such as light sensitivity, heat resistance, conductivity, corrosion resistance and physiological action. PAHs possess very characteristic UV absorbance spectra. Each ring structure has a unique UV spectrum, thus each isomer has a different UV absorbance spectrum. This is especially useful in the identification of PAHs. Most PAHs are also fluorescent, emitting characteristic wavelengths of light when they are excited (when the molecules absorb light).

PAHs are usually found as a mixture containing two or more of PAH compounds, such as soot. The major source of PAHs is the incomplete combustion of organic material such as coal, crude oil, garbage, tobacco and wood. High temperature cooking also forms PAHs in meat and in other foods. PAHs are not synthesized chemically for industrial purposes.

The sources of PAHs can be both natural and anthropogenic.

**Natural sources** include:
- Volcanoes
- forest and grass fires
- Agricultural burning
- oil seeps
- chlorophyllous plants, fungi, and bacteria

**Anthropogenic sources** of PAHs include:
- refuse incineration
- petroleum
- Road transport
- Power plants
- Domestic burning of coal and wood
- Fossil fuel burning
- production of coke, dyes, carbon black, coal tar, and pesticides.
It is important to mention that the incomplete combustion, either naturally or anthropogenically derived, has been identified as the single largest contributor of PAHs to the environment\textsuperscript{137}.

Commercial uses for many PAHs include, as intermediaries in agricultural products, pharmaceuticals, photographic products, plastics, lubricating materials, and other chemical industries\textsuperscript{135}.

The general uses of targeted PAHs are:

- **Anthracene**: manufacture of dyes and pigments, and diluent for wood preservatives.
- **Phenanthrene**: manufacture of pesticides and resins.
- **Pyrene**: manufacture of pigments.
- **Fluoranthene**: manufacture of dyes, pharmaceuticals and agrochemicals.

Other PAHs may be contained in asphalt used for the construction of roads, in addition to roofing tar and also in the field of electronics, functional plastics, and liquid crystals\textsuperscript{135}.

PAHs enter the environment mostly as releases to air from volcanoes, forest fires, residential wood burning, and exhaust from automobiles and trucks. They can also enter surface water through discharges from industrial plants and waste water treatment plants, and they can be released to soils at hazardous waste sites if they escape from storage containers. Contamination of water may take place through asphalt or coal tar coating of storage tanks and water distribution pipes are used. Thus, PAHs are commonly detected in air, soil, and water. Therefore, PAHs are considered ubiquitous in the environment\textsuperscript{138-139}.

PAHs are a concern because they are persistent. Because they do not burn very easily, they can stay in the environment for long periods of time. Individual PAHs vary in behavior. Some can turn into a vapor in the air very easily. Most do not break down easily in the water.

As stated above in section 1.3.2, four polyaromatic hydrocarbons (PAHs) namely, anthrance (Anth), fluoranthene (Flu), pyrene (Pyr), and phenanthrene (Phen) are of concern in this Ph.D. thesis and are present in the list of 16 priority PAHs of U.S. EPA (Environmental Protection Agency). The physico-chemical properties of four targeted PAHs are summarized in Table 1.4.
**Table 1.4 | Physicochemical properties of targeted PAHs.**

<table>
<thead>
<tr>
<th>Property</th>
<th>Anthracene</th>
<th>Phenanthrene</th>
<th>Pyrene</th>
<th>Fluoranthene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C(<em>{14})H(</em>{10})</td>
<td>C(<em>{14})H(</em>{10})</td>
<td>C(<em>{16})H(</em>{10})</td>
<td>C(<em>{16})H(</em>{10})</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>178.23</td>
<td>178.23</td>
<td>202.25</td>
<td>202.26</td>
</tr>
<tr>
<td>Form/colour</td>
<td>Yellow crystals</td>
<td>colourless</td>
<td>Colourless solid</td>
<td>Pale yellow crystals</td>
</tr>
<tr>
<td>Odour</td>
<td>Weak aromatic</td>
<td>Faint aromatic</td>
<td>Faint aromatic</td>
<td>Aromatic</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>218</td>
<td>101</td>
<td>150.62</td>
<td>111</td>
</tr>
<tr>
<td>Boiling Point (°C)</td>
<td>342</td>
<td>340</td>
<td>404</td>
<td>384</td>
</tr>
<tr>
<td>log K(_{ow})</td>
<td>4.45</td>
<td>4.46</td>
<td>4.88</td>
<td>5.16</td>
</tr>
<tr>
<td>Water solubility (25°C)</td>
<td>0.0434 mg/L</td>
<td>1.15 mg/L</td>
<td>0.135 mg/L</td>
<td>0.26 mg/L</td>
</tr>
</tbody>
</table>

*Source: HSDB (Hazardous Substances Data Bank, U.S.)*

Anthracene is one PAH that is used as an important source for manufacturing anthraquinone and alizarin dyes for cotton fibers. On the other hand, the industrial production of hydrogen peroxide also requires the presence of anthracene as a raw material. In these cases the determination of anthracene in residual water can be important for monitoring its environmental impact.

Phenanthrene is a priority PAH, and has been shown to have high toxicity to marine diatoms, mussels, gastropods, crustaceans and fish. It has been detected in surface water and has already become the subject of various scientific fields. Results of toxicity tests showed that PHE is toxic to aquatic organisms, so it may impose potential risks to the ambient aquatic environment.
Of the 16 PAHs, pyrene occurs at relatively high concentrations in PAH mixtures and is also one of the most highly concentrated PAHs detected in drinking water\textsuperscript{146-147}.

Fluoranthenone contains three benzene rings in molecular structure and is one of the polycyclic aromatic hydrocarbons (PAHs) and an environmental persistent organic pollutant and is currently being routinely monitored for regulatory purposes\textsuperscript{148}. Water Framework directive of the European Union includes six PAHs, including fluoranthene (as an indicator of other, more dangerous, polyaromatic hydrocarbons) in a list of priority substances of significant environmental risk which must undergo progressive reduction in emissions and disposal into the aquatic environment\textsuperscript{149}.

1.3.6.1 Exposure

Humans are exposed to PAH by inhaling contaminated air, by ingesting tainted food, drinking and source waters, by non-dietary ingestion of contaminated dust or soil, or less significantly in non-occupational settings, by dermal absorption from environmental media. The exposure resulting from ingestion of dust or soil is believed to be more important for young children because of their play activities. Exposure to PAH via the inhalation pathway is due to PAH present in ambient and indoor air. In inner cities, these PAH concentrations are higher than in rural areas, because of heavier traffic and more industrial sources\textsuperscript{150}. Significant indoor PAH contamination is also from the presence of tobacco smoke and combustion heating appliances\textsuperscript{151}. Exposure to PAH through the nondietary ingestion pathway is due to PAH in house dust, in play area soil, or on other household surfaces. The PAH exposure through the dietary ingestion pathway is from the PAH present in foods and drinking water. Foods having significant levels of PAH include charcoal-broiled or smoked meats, leafy vegetables, grains, fats, and oils, all typically having PAH concentrations of tens of ppb\textsuperscript{152}.

The main source of PAH contamination in drinking-water, which is also major source of exposure, is usually not the source water but the coating of the drinking-water distribution pipes. At least in the past, coal tar was a common coating material for water pipes, used to give effective protection against corrosion. After the passage of drinking-water through those pipes or after repair work, there is a significant increase in PAH levels, detected in the water\textsuperscript{153-154}. Although WHO has called for a cessation of this practice (WHO, 1996), many countries still have a large amount of pipes lined with coal tar coating\textsuperscript{155}. 


Contamination of environmental water which is widely used throughout the world for many purposes including agricultural and domestic purposes, is mainly from highly industrially polluted rivers, the concentrations of individual PAHs in surface and coastal waters are generally 50 ng/litre. Concentrations above this level (sometimes into the 10 μg/litre range) indicate contamination by PAHs mainly through industrial point sources and atmospheric deposition, and urban runoff.\textsuperscript{156}

1.3.6.2 Toxicological Profile

PAHs are reasonably anticipated to be human carcinogens. There are a number of occupational epidemiologic studies that show increased incidence of cancer in humans exposed to mixtures of PAHs by inhalation or dermal contact e.g. the first report of increased incidence of scrotal cancer among chimneysweepers by Sir Percival Pott in 1775. After this pioneer study, different epidemiological studies pointed out the high incidence of tumors in workers exposed to cigarette smoke, coke oven emissions and roofing-tar emissions and cigarette smoke.\textsuperscript{157-159}

Occupational exposures to high levels of pollutant mixtures containing PAHs has resulted in symptoms such as eye irritation, nausea, vomiting, diarrhoea and confusion. Mixtures of PAHs are also known to cause skin irritation and inflammation. Health effects from chronic or long-term exposure to PAHs may include decreased immune function, cataracts, kidney and liver damage (e.g. jaundice), breathing problems, asthma like symptoms, and lung function abnormalities, and repeated contact with skin may induce redness and skin inflammation.\textsuperscript{160}

1.3.6.3 Guideline and regulation

Polynuclear aromatic hydrocarbons (Total) in drinking water- 0.000 1 mg/L.\textsuperscript{161}

1.3.7. DI-2-ETHYLHEXYL PHthalate

Reasonably anticipated to be a human carcinogen,

Also known as DEHP, diethylhexyl phthalate, or dioctyl phthalate.

Di (2-ethylhexyl) phthalate (DEHP) is a typical and widely produced phthalate, widespread in the environment and a highest occurring synthetic chemical, in drinking water.\textsuperscript{162}

1.3.7.1 Properties and uses

DEHP is a phthalate ester that exists as a colorless oily liquid with a slight odor. It is slightly soluble in water and carbon tetrachloride, miscible with mineral oil and hexane, and soluble in blood and body fluids containing lipoproteins. Physical and chemical properties of DEHP are listed in the following Table 1.5.
## Table 1.5 | Physicochemical properties of DEHP. *

<table>
<thead>
<tr>
<th>Property</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C_{24}H_{38}O_{4}</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>390.56</td>
</tr>
<tr>
<td>Form/colour</td>
<td>Colourless oily liquid</td>
</tr>
<tr>
<td>Odour</td>
<td>Slight odour</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>-55</td>
</tr>
<tr>
<td>Boiling Point (°C)</td>
<td>384</td>
</tr>
<tr>
<td>log K_{ow}</td>
<td>7.60</td>
</tr>
<tr>
<td>Water solubility (25°C)</td>
<td>Sparingly soluble in water (0.27 mg/L)</td>
</tr>
</tbody>
</table>

Chemical structure

*Source: HSDB (Hazardous Substances Data Bank, U.S.)*

The uses of DEHP fall into two major categories:

- Polymer uses
- Non-polymer uses

About 97% of DEHP produced is used as a plasticizer in polyvinyl chloride (PVC) resins for fabricating flexible vinyl products\textsuperscript{163-164}. As a plasticizer, the primary function of DEHP is to soften otherwise rigid plastics and polymers. Products typically contain from 1% to 40% DEHP. The DEHP levels in PVC medical tubing may be as high as 80%\textsuperscript{165}. Plasticized PVC has been used in many consumer items and building products, such as furniture, tablecloths, shower curtains, and leather, floor tiles, swimming-pool liners, wire and cable, rainwear, shoes, toys, dolls, baby pants, food packaging materials, tubing used in commercial milking equipment, and weather stripping\textsuperscript{166-167}. DEHP is also used in medical devices (blood and intravenous solution bags, catheters, tubing for dialysis and parenteral solutions, oxygen masks, and urine and colostomy bags) and in disposable surgical gloves. It has been used as a plasticizer in non-PVC materials, including polyvinyl butyral, natural and synthetic rubber, chlorinated rubber, ethyl cellulose, and nitrocellulose\textsuperscript{166}.
Non-polymeric uses of DEHP include its use in adhesives, inks, cosmetics, munitions, perfumes, paints, additives in hair-sprays, in dielectric fluids for electric capacitors, as an inert ingredient in pesticides, insect repellents, in lubricating oil, to detect leaks in respirators and in testing air-filtration systems\(^{167-168}\).

### 1.3.7.2 Occurrence and Exposure

Di (2-ethylhexyl) phthalate is not known to occur naturally. DEHP is found in many environmental matrices, such as drinking water, ground water, surface waters, wastewaters, landfill leachate, sludge, soil, and sediments\(^{163, 169-174}\). Indoor and outdoor end products of PVC including plastic water bottles, plastic water bags, are important sources of DEHP pollution in water and soil. DEHP can be primarily released from indoor and outdoor products to the soil compartments, and a fraction may ultimately be distributed to the air or surface water compartments. During the whole life cycle of products containing DEHP, around 72% of DEHP is released into the soil, 21% ended up in water, and the rest 7% is evaporated into the air\(^{175}\). Moreover, DEHP is released into the environment during its production, transportation, manufacturing, and improper disposal\(^{176}\).

Further, because of the widespread use of DEHP in plastic food containers and plastic water bottles, and its ability to leach out of PVC, humans are exposed to this substance on a daily basis. The extensive manufacture of DEHP containing plastics has resulted in its becoming a ubiquitous environmental contaminant\(^{176-178}\). The general population studies show that nearly all human population absorb DEHP and excrete its metabolites in their urine in measurable amounts and concentrations in body fluids vary substantially, and exposure is on higher side.

Occupational exposure to DEHP rose to prominence, i.e. in human blood stored in PVC bags\(^{179}\). Later in 972, the same authors later reported the presence of DEHP in tissue samples of the lung, liver and spleen from patients who had received blood transfusions\(^{180}\). While occupational inhalation is a significant potential route of exposure, medical procedures such as blood transfusion, haemodialysis, extracorporeal membrane oxygenation, umbilical catheterization and short-term cardiopulmonary bypass can also result in high exposure\(^{177,181}\). Patients undergoing haemodialysis are considered to have the highest exposure, due to the chronic nature of the treatment.

### 1.3.7.3 Toxicological Profile

A major concern is DEHP’s effect on the development and reproductive system. The male reproductive toxicity of DEHP is well established in laboratory animals.
including rats, mice, hamsters, and ferrets. Depending on the dose, duration of exposure, and age of animals, DEHP causes reduced fertility, decreased weights of male reproductive organs, and histopathological changes in the testes of adult rats. DEHP also has been found to cause developmental toxicity, including death, developmental delay, and structural malformations and variations; neurological developmental effects have also been reported. Evidence of DEHP’s reproductive toxicity in humans is less conclusive.

According to U.S. EPA, there is sufficient evidence that DEHP causes cancer in laboratory animals. DEHP is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals. However, the data available from epidemiological studies are inadequate to evaluate the relationship between human cancer and exposure specifically to DEHP (IARC 1982). Neurological effects such as alteration in brain function and behavioural changes has been observed in rodent studies. The results thus indicated that DEHP might have potential effects on neural development and behavior. There is some evidence in laboratory animals indicating that DEHP may affect the thyroid and immune system. Little information is available on its effect on the human thyroid and immune system.

1.3.7.4 Guidelines and regulations

The EPA limits the amount of DEHP that may be present in drinking water is 6 ppb, or 6 ug/L. The Occupational Safety and Health Administration (OSHA) sets a maximum average of 5 mg/m$^3$ of air, in the workplace during an 8-hour shift. The short-term (15-minute) exposure limit is 10 mg/m$^3$. Listed as a potential occupational carcinogen.

1.4 ANALYTICAL METHODOLOGY

The primary goal of this Ph.D. study is to innovate novel, green analytical protocols for trace analysis of toxic substances, using chromatographic technology and to successfully apply them to real world water samples.

It is stating a stereotype to say that analytical methodology is a driving force of scientific progress. The analytical methodology strives to provide accurate, precise and validated data to support the developed analytical methods. Most will agree, too, that the birth of a new analytical protocol/method comes only with the painstaking accumulation of optimization and validation. In targeted chromatographic measurement, the goal is set before analysis: “we wish to determine substance X in matrix Y”. Next, a
method is selected or developed to provide a quantitative measurement. The procedure has three steps: (1) sample preparation, which consists of extraction, clean up and optional derivatization; (2) chromatographic separation, and (3) detection. All three contribute to selectivity and sensitivity.

Analytical chemistry is a multistep endeavour, measurement is the final link at the end of a chain of operations that begins with sample preparation. The quantification of low concentrations of toxics, is not possible, due to presence of other matrix chemical species present in the sample; these could interfere with the analysis. Therefore, to avoid quantification artefacts, other matrix chemical species need to be separated and analyte/analytes of interest, needs to be enriched before chromatographic analysis. Here comes the role of sample preparation/pretreatment techniques. The sample preparation/pretreatment is an essential process that underlie all subsequent work, and impart relevance to what would otherwise be a meaningless exercise. In all of the diverse forms of analysis, it is important to recognize that sample preparation is part of the whole analytical workflow. This is especially true of our study, without sample preparation the trace analysis of toxics in, whatever the water matrices considered, is not possible because matrix elements immediately contaminate the matrix, as many compounds are/may present in different water matrices. Also it is clear that errors made in sampling, sample preparation or sample introduction (injection) cannot be corrected by using even the most advanced chromatographic systems. In this Ph.D. study, there have been two major trends: miniaturization, and higher sensitivity. Miniaturization can also help make sample preparation “greener” through solvent reduction or even elimination. Following the increase in sensitivity of analytical protocols, sample preparation has the same importance but can be miniaturized or adapted to be greener. Such approaches are typically well used in this study.

Additionally, this analytical study strives to be at the forefront of method development, using classical solid phase extraction technique (SPE), novel sample preparation microextraction techniques such as microextraction in packed syringe (MEPS), and fabric phase sorptive extraction (FPSE), that augments the toxic analysis and advances the analysis by providing analytical advances such as lower detection limits, quantification of ultra-trace level (ng/pg) and application of the analytical methodology to real water samples.
On chromatographic platform, high performance liquid chromatography (HPLC) coupled with ultra-violet (UV) and fluorescence detector (FLD) have been used in this study, offers the benefit of compound specific analysis and low detection limits (ng/mL or/and pg/mL).

1.4.1 Technological platforms for sample preparation

As stated above, in this study three sample preparation techniques including classical SPE, with two novel, miniaturised techniques, MEPS and FPSE have been applied successfully, for the trace analysis of targeted toxic substances in water matrices, and have been discussed in the following paragraphs.

1.4.2 Solid phase extraction (SPE)

Solid phase extraction (SPE) is a form of step-wise chromatography designed to extract, adsorb and/or partition, one or more components from a sample (liquid phase) onto a stationary phase (solid sorbent). SPE is the most fundamental and powerful technique available for the rapid and selective sample preparation prior to analytical chromatography. SPE extends the lifetime of chromatographic systems and improves qualitative and quantitative analysis. By switching sample matrices from the original complex matrix to a simpler matrix environment, subsequent analysis is often simplified, and the demand placed on an analytical system is considerably lessened. Thus solid phase extraction (SPE) is a very useful technique for sample preparation due to its convenience, effectiveness, relatively low solvent usage and versatility.

The first reported use of Solid Phase Extraction (SPE) in 1974 by R. Adams and co-workers\(^ {184}\), SPE uses solid particle, chromatographic packing material, usually contained in a cartridge type device, to chemically separate the different components of a sample. Samples are nearly always in the liquid state. The chromatographic bed can be used to separate the different compounds in a sample, to make subsequent analytical testing more successful. For example, SPE is often used for the selective removal of interferences.

The technically correct name for this technology is “Liquid-Solid Phase Extraction,” since the chromatographic particles are solid and the sample is in the liquid state. The same basic chromatographic principles of liquid chromatography that are used in HPLC are also used here, but in a different format and for a different reason.
Here, chromatography is used to better prepare a sample before it is submitted for analytical testing. SPE can be used with a variety of sample matrices such as environmental (water, soil), biological (blood, saliva, or urine) and food.

SPE involves partitioning between a liquid (sample matrix) phase and a solid (sorbent) phase whereby the intermolecular forces between the phases influences retention and elution. The wide range of SPE sorbents available such as butyldimethyl bonded silica (C4), octyl bonded silica (C8), octadecyl bonded silica (C18), Strong cation exchange (sulfonic acid bonded silica with Na+ ) (SCX) etc., provides a wide range of interactions. This sample treatment technique enables the concentration and purification of analytes from solution by sorption on a solid sorbent. The basic approach involves passing the liquid sample through a column, a cartridge, a tube or a disk containing an adsorbent that retains the analytes. After the entire sample has been passed through the sorbent, retained analytes are subsequently recovered upon elution with an appropriate solvent.

Ideally, an SPE method always consists of four successive steps (Figure 1.2). First, the solid sorbent should be conditioned using an appropriate solvent, followed by the same solvent as the sample solvent. This step is crucial, as it enables the wetting of the packing material and the solvation of the functional groups. In addition, it removes possible impurities initially contained in the sorbent or the packaging. Also, this step removes the air present in the column and fills the void volume with solvent.

![Figure 1.2 | Schematic diagram of Solid phase extraction (SPE) procedure.](image-url)
The second step is the loading of the sample through the solid sorbent. During this step, the analytes are concentrated on the sorbent. Even though matrix components may also be retained by the solid sorbent, some of them pass through, thus enabling some purification of the sample. The third step is the washing of the solid sorbent with an appropriate solvent, having low elution strength, to eliminate matrix components that have been retained by the solid sorbent, without eluting any analyte. A drying step may also be advisable, especially for aqueous matrices, to remove traces of water from the solid sorbent.

Figure 1.3 | SPE manifold (A) and cartridges (B) in our research lab.

The final and fourth step consists of elution of the analytes of interest by an appropriate solvent, without removing retained matrix components. The solvent volume should be adjusted so that quantitative recovery of the analytes is achieved with subsequent low dilution.

SPE is probably the most widely adopted technique for preparing samples in the analysis of pharmaceuticals, toxic chemicals and drugs of abuse in environmental and biological samples. The large variety of sorbents commercially available makes this technique suitable for the determination of analytes with divergent chemical structures and polarities.

The SPE instrument i.e. SPE manifold used in this Ph.D. research work is shown in Figure 1.3.
1.4.3 Microextraction by packed sorbent (MEPS)

MEPS is a relatively new and a different approach to sample preparation. It was developed by Mohamed Abdel Rehim in 2004\textsuperscript{188}. MEPS is basically a miniaturized SPE that performs the same function as SPE, namely the purification or pretreatment of samples, but with some significant differences\textsuperscript{189}.

- MEPS works with much smaller samples (as small as 10µL) than full scale SPE.
- MEPS can be easily semi-automated and fully automated.
- MEPS is applicable to GC and LC.
- The sample processing, extraction and injection steps are performed using the same syringe and eluted extract is directly injected to LC or GC.
- Significantly reduces the volume of solvents and sample needed.
- A green sample preparation technique.

Basic principle of MEPS is same as of SPE, with a manual modification in MEPS i.e. cartridge is integrated into the syringe (Figure 1.4 A) which ease the handling and make the sample treatment procedure, more precise and fast. MEPS is essentially a miniaturized SPE column in a syringe with smaller bed dimensions i.e. amount of sorbent is 0.5-2 mg as compared to 50-2000 mg in SPE. So MEPS may be applied to all SPE methods by scaling down the amount of reagents, solvents and sample volumes.

The real strength of MEPS is that the entire concentrated sample extract is down to elution volume for analysis rather than a portion, which inturns make this technique remarkably sensitive, with much lower detection limits. Second point is that MEPS draws and elutes from the bottom, Figure 1.4 B. The double pass of the sample reduces the weakly bound fraction of the sample and thus significantly improves the sample clean up process.

Various sorbent materials such silica based i.e. butyldimethyl bonded silica (C\textsubscript{4}), octyl bonded silica (C\textsubscript{8}), octadecyl bonded silica (C\textsubscript{18}), C\textsubscript{18} + SCX (strong cation exchange, sulfonic acid bonded silica) and/or molecular imprinted polymers (MIPs) are packed in MEPS BIN (barrel and insert needle assembly, Figure 1.4 C) and are used for various analytes\textsuperscript{187-190}. 
Figure 1.4 | Microextraction by packed sorbent (MEPS).
1.4.4 Fabric phase sorptive extraction (FPSE)

Fabric phase sorptive extraction is a new generation green sample preparation approach developed by A. Kabir et al. in 2014. This most recent member of the sorptive microextraction family has innovatively incorporated both solid phase extraction (SPE) and solid phase microextraction (SPME) technique into a single technology platform. FPSE utilizes permeable natural/synthetic fabrics e.g., cotton, polyester, fiber glass and cotton-polyester supports to chemically bind sol-gel hybrid organic-inorganic sorbents. A 5 cm, 2 unit of coated fabric (2.5 cm x 2.0 cm) is typically used as the extraction media which can be inserted directly into the sample container (Figure 1.5). The high primary contact surface area and the open geometry of FPSE fiber combined with sol-gel derived sponge-like porous sorbent in the form of ultra-thin coating enable the rapid sorbent-analyte interaction so that target analyte(s) can be extracted from complex sample matrices at high efficiency in a fraction of time compared to its conventional analogs. Extraction of the analytes is generally expedited by using a magnetic stir bar to diffuse the analytes into the sample matrix so that rapid mass transfer of analytes of interest, from the sample matrix to the extraction medium takes place. Once the mass transfer equilibrium between the FPSE media and the sample matrix is reached, the FPSE media is removed from the sampling container and dried. Subsequently, the FPSE media is exposed to a small volume of organic solvent to elute/back-extract the preconcentrated analytes of interest. The prepared sample in a suitable organic solvent can then be injected into the analytical instrument for analysis.

FPSE is an equilibrium extraction strategy. However, compared to SPE and SPME, FPSE uses high mass of sorbents. In addition FPSE exploits its quasi flow-through extraction media that allows the sample matrix passing through it and helps acquiring near-exhaustive extraction under equilibrium extraction condition in a relatively short period of time. The migration of aqueous sample matrix through the extraction media speeds up the mass transfer from the sample matrix to the extraction media, leading to high absolute recovery of analytes in a short period of time. Subsequently, a small volume of organic solvents can easily access to the interaction sites of the sorbent to break sorbent analyte interaction and quantitatively elute/back-extract the analytes of interest. FPSE C_{18} Fiber used in this Ph.D. research work is shown in Figure 1.6.
Figure 1.5 | Schematic diagram of FPSE.

Figure 1.6 | FPSE fibers (C₁₈) used in the research work.
FPSE has numerous advantages:

- FPSE is a green sample preparation approach.
- Hybrid inorganic-organic sorbents demonstrate high chemical, mechanical, chemical solvent stability.
- Completely eliminates the solvent evaporation/sample reconstitution step, which is a time consuming, environment unfriendly and error prone step.
- High sorbent loading results into high sample capacity, and wide range of analytes linearity.
- Unlike inert supports used in SPE/SPME, the fabric support used in FPSE complements the ultimate polarity of the FPSE media through hydrophobic or hydrophilic property of the substrate used.
- Permeable and sponge-like porous sol-gel sorbent ensures fast and maximum extraction equilibrium.
- Both Extraction modes are possible with FPSE i.e. equilibrium extraction (like SPME) and exhaustive extraction (like SPE).
- Complex samples containing particles, debris, biomass, proteins, cells can be directly used and does not require any matrix clean-up (e.g., filtration, centrifugation etc.).
- Chemical bonding between the substrate and the sorbent allows employing any organic solvent of choice for analytes elution/back-extraction after the extraction. As a result, the prepared sample can be analyzed in multiple analytical systems (GC/HPLC/CE) for complementary information.
- Due to the integration of SPE and SPME in FPSE, any sorbent used in both the techniques can be used FPSE. This opens up the possibility of utilizing hundreds of different sorbents used in both the techniques.

Till now, FPSE has been applied in the isolation of various types of analytes such as antibiotics in milk, herbicides in environmental waters, non-steroidal anti-inflammatory drugs in environmental water samples, estrogens in water and benzodiazepines in blood serum, endocrine disruptors e.g. alkyl phenols from ground water, river water, sewage water, sludge and soil samples.
1.4.5 High performance liquid chromatography (HPLC)

Back in 1941, A. H. Gordon et al., published a paper predicting that high pressure and small particles will lead to efficient separations\textsuperscript{195}. But, it took another 25 years, to publish the first paper of HPLC, in 1966, by Csaba Horváth et al., described the ion-exchange separation of nucleotides and thyroid compounds\textsuperscript{196}. The first commercial HPLC instrument was brought to the market by Waters Association after one year\textsuperscript{197}. The so called ALC-100 was formally introduced at the 1968 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pitcon) in Cleveland, USA and the first HPLC meeting was held in Interlaken, Switzerland in 1973.

High-performance liquid chromatography (HPLC) is an analytical technique to separate, identify, and quantify components in a mixture. It is the single biggest chromatography technique essential to most laboratories worldwide. HPLC has proven its potential in separation science as a reliable and heavily used technique, with superior performance and wide applicability in many different fields. Over the years, HPLC has become a well-established separation technology that is continuously evolving to face the challenges posed by research and industry, as well as regulatory agencies, and to meet, the even more stringent requirements for qualitative and quantitative analysis, in terms of speed, accuracy, and sensitivity.

To date, HPLC has advanced to be, the analytical determination method of choice, for environmental, clinical, water, forensic, and pharmaceutical fields. HPLC could be regarded as the central standardization workhorse, in many analytical or manufacturing laboratories. Nowadays, HPLC equipment is very sophisticated and reliable, and it comes with different types of detector and autosamplers as well as computers that control operation, processing, data storage, and retrieval.

Chemical analysis of toxics in water, is necessary to ensure quality control and consumer safety. As a result, the employment of qualitative or quantitative chromatographic techniques such as high-performance liquid chromatography (HPLC) could be very useful, for quality control of water. Furthermore, HPLC analytical techniques have been included in the latest monographs on the identification and determination of the toxics in different matrices i.e. food and water\textsuperscript{198-204}. Using HPLC, avoids the main problems associated with the use of gas chromatography such as their high molecular mass, polarity, thermal stability of components, and compound destruction.
1.4.5.1 HPLC as an analytical Tool

**Principal working**

In classical column chromatography a solvent drips through a column filled with an adsorbent (mostly silica) under gravity. HPLC is a highly improved version of column chromatography. In HPLC, a pump forces a solvent, or a mixture of solvents through a column under high pressures of up to 400 atmospheres. The column packing material or adsorbent called stationary phase is typically a fine granular material made of solid particles such as silica and/or polymers. As shown in the schematic diagram in **Figure 1.7**, HPLC instrumentation includes a solvent reservoir, pump, injector, column, detector and data display system. The heart of the system is the column where separation occurs.

The high pressure makes the technique much faster compared to classical column chromatography and thus also called high pressure liquid chromatography. This allows using much fine, smaller particles for the column packing material. The smaller particles have a much greater surface area which allows, better interactions between the stationary phase and the molecules flowing past it. This results in a much better separation of the components of the mixture. The pressurized liquid is typically a mixture of solvents such as acetonitrile, methanol and water, and is referred to as the mobile phase. The components of a mixture (analytes) are separated from each other due to their different degrees of interaction with the stationary phase. This causes different elution rates for the different components and leads to the separation of the components as they flow out of the column.

The chromatographic process begins by injecting the solute into the injector at the end of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a peak on the data display. Detection of the eluting components is important, and the method used for detection is dependent upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyze the chromatographic data, integrators and other data-processing equipment are frequently used. Compared to column chromatography, HPLC is highly automated and extremely sensitive.
1.4.5.2 Basic concepts of HPLC

Retention: The retention of an analyte with a stationary phase and eluent (mobile phase) is expressed as a retention time. Retention time is defined as how long a component (analyte) is retained in the column by the stationary phase relative to the time it resides in the mobile phase. The retention is best described as a column capacity factor ($k'$), which can be used to evaluate the column efficiency. The longer a column retains a component, the greater is its capacity factor. The column capacity factor of a compound (A) is defined by the equation:

$$k = \frac{T_A - T_O}{T_O}$$

where $T_A$ is the retention time of the analyte peak, and $T_O$ is the dead time (retention time of system peak) of the column.

Resolution: Resolution is the ability of the column to separate peaks on the chromatograph. Resolution (R) is expressed as the centre to centre separation between two peak maxima to the mean value of the peak width at the base line. It is expressed as the equation:

$$R = \frac{(T_B - T_A)^2}{W_A + W_B}$$

where $T_A$ is the retention time of component A, $T_B$ is the retention time of component B, $W_A$ is the peak width of component A and $W_B$ is the peak width of component B.

If $R \geq 1$, then components are completely separated, but if $R \leq 1$, then components overlap.
**Sensitivity**: Sensitivity is a measure of the smallest detectable level of an analyte in a chromatographic separation and is dependent on the signal-to-noise (S/N) ratio in a given detector. Sensitivity can be enhanced, by derivatization of analyte, and optimization of chromatographic conditions (mobile phase, flow rate, wavelength).

The versatility of HPLC in analytical separation is mainly due to the availability of numerous chromatographic variants (normal phase, reversed-phase, ion-exchange, ion-pair, and others), its reproducibility (peak performance and results), and its selectivity (compounds of specific moieties/structures). The two most common variants used, are normal-phase and reversed-phase HPLC.

1.4.5.3 Normal-Phase HPLC

The column is filled with tiny fine silica particles, and a non-polar solvent, for example, hexane. A typical column has an internal diameter of 4.6 mm or smaller and a length of 150 to 250 mm. Non-polar compounds in the mixture will pass more quickly through the column, as polar compounds will stick longer to the polar silica than non-polar compounds.

1.4.5.4 Reversed-Phase HPLC

The column size is the same. The column is filled with silica particles which are modified to make them non-polar. This is done by attaching long hydrocarbon chains (C₈, C₁₈) to its surface. A polar solvent is used, for example, a mixture of water and an alcohol such as methanol. Polar compounds in the mixture will pass more quickly through the column because a strong attraction occurs between the polar solvent and the polar molecules in the mixture. Non-polar molecules are slowed down on their way through the column. They form varying degrees of attraction with the hydrocarbon groups principally through *van der waals* dispersion forces and hydrophobic interactions. They are also less soluble in the aqueous mobile phase components facilitating their interactions with the hydrocarbon groups. Reversed phase HPLC is the most commonly used form of HPLC.

1.4.5.5 Columns

The HPLC column is filled with very small particles (also called gels), and the size of gel is 3 to 15 μm. This gel has various "traps". Each sample component has different "characteristics" and interacts with the "trap" differently, i.e., each component may stay inside the column for different lengths. Thus using this time differences, the
components are separated. There are different HPLC column packing materials (base material) available. Moreover using different modifications (addition of chemical compounds on the surface of the gel), a wide variety of HP LC column can be prepared.

1.4.5.6 Packing materials in column (Types of gels)

Silica gel is the most popularly used packing material. Silica, silicon dioxide, has the chemical formula of SiO$_2$. You may see a small paper bag of silica in food packages stated 'do not eat'. It is used as a dehydrator. The ones used for dehydrator has a gel diameter of 1 mm or larger, but the ones packed in LC columns are very small; few um sizes. There are two types of silica gels. The one has spherical shapes, the other has irregular shapes. Unlike past, the spherical shaped gels are most widely used these days. The silica gel used in LC has pores on the surface of the gel. By having the pores, it provides larger surface area compared to the ones without pores. The size of pore is very small and expressed in angstrom (Å) unit. The silica with pores is called porous silica.

1.4.5.7 There are few indexes used to express silica gel grades.

Shape: Most silica columns used nowadays contain spherical type.

Size: Smaller size particles have been developed. Current major line is 5μm, but even smaller size 1.5 to 3μm gel is also in use. The smaller gels are packed in smaller column housing and thus decreases the analytical time.

Pore size: There is not a simple good/bad indicator for pore sizes. The right pore size should be determined depending on the size of target analyte.

Surface area: This is the relative surface area of the gel. The smaller the particle size, the relative surface area becomes larger. Also the larger the number of pores, the larger the relative surface area. If all the other indexes are the same, the better performance can be expected from the larger surfaced-area gel. One gram of conventional silica gel provides a surface area of softball field.

In the earlier stage of HPLC development, almost always silica gels were used. However, polymer-based column is becoming popular. The generally known polymers include polyethylene and poly propylene. Similar to the silica gel, the polymer gel is manufactured into very small particles.

Other than silica and polymer gels, the gels used include natural substances such as cellulose, agarose, dextrin, and chitosan, and members of ceramics such as hydroxylapatite and zirconia. However, their use is very limited.
In this Ph.D. research work C\textsubscript{18} columns of two dimensions (Figure 1.8) have been used:

- Reverse phase C\textsubscript{18} column (25 cm × 4.6 mm, particle size 5 µm) c(Merck Purospher\textsuperscript{R} STAR and Dionex Acclaim 120).
- Reverse phase C\textsubscript{18} column (10 cm × 4.6 mm, particle size 5 µm) (Acentis Express, Supelco).

![HPLC C\textsubscript{18} columns used in the research work.](image)

**1.4.5.8 Detectors**

The various detectors, such as photodiode-array detectors (PDA), fluorescence detectors, electrochemical detectors, and mass-spectrometers, that are used to identify and quantify column throughputs have only enhanced the power of HPLC in the quality control and standardization of natural products. Ultraviolet detectors are the primary means of detection in the HPLC analysis of alkaloids and adulterants. Fixed wavelength (254 and/or 280 nm) and multiwavelength detectors like PDA have been widely employed with sensitivity in the nanogram range or even lower. HPLC-MS has revolutionised the study of plant alkaloids as it also provides information on the possible structure and molecular weight of the parent alkaloid or its metabolites.

HPLC, with its advanced, reliable column- and detector-technologies, will remain the central analytical tool for environmental, water, forensic and pharmaceutical research.
In this piece of study, HPLC has been used successfully, coupling with UV and FLD detectors respectively. The HPLC-UV/FLD instrument used in the study, in our lab is shown in **Figure 1.9**.

![HPLC system in the research lab.](image)

**Figure 1.9 |** HPLC system in the research lab.

### 1.4.6 Method validation: Definitions and terminology

The following paragraphs present a discussion of the characteristics, for consideration during the general validation process of the analytical methods/procedures. Furthermore, this text serves as a collection of terms, and their definitions, well-engaged in analytical validation process.

An indispensable component to successful analytical protocol/method qualification is its analytical validation, which is also a regulatory requirement. The objective of analytical validation is to demonstrate its suitability for the intended purpose and to obtain accurate, consistent and reliable data. Validation of an analytical protocol/method consists of analysing or verifying various analytical parameters as described in the ICH guidelines\(^1\) (International Conference of Harmonisation). A comprehensive analytical method validation consists of a huge amount of laboratory work. Within a method validation process, the performance of an analytical method are studied by extensive laboratory testing of different parameters like specificity, linearity,
range, accuracy, precision, detection limit, quantification limit and robustness, information about the possible errors, and their quantification within the method.

In this Ph.D. thesis analytical research work, when designing an analytical validation procedure, particular attention has been given to the intended purpose of the method by defining the critical parameters that need to be validated.

**The specific definitions of analytical validation parameters are briefly described below:**

- **Specificity** (or selectivity) is an analytical parameter, which describes the ability of an analytical method to assess (measure) unequivocally the analyte in the presence of other components in a given sample, which may be expected to be present in the sample (e.g. matrix, impurities). Specificity in high performance liquid chromatography is obtained by choosing optimal columns and setting optimal chromatographic conditions, such as mobile phase composition, detector wavelength and column temperature. In addition to it, the sample preparation step is also optimized for best specificity.

- **Linearity** describes the ability of an analytical method to elicit test results (within a given range), which are directly, or by a well-defined mathematical transformation, proportional to the concentration (amount) of an analyte in a sample. In this thesis, linearity has been evaluated by calculation of a regression line by the method of least squares, with its correlation co-efficient, slope and Y intercept.

- **Range** of a method is the interval between the lower and upper limit of concentrations of analyte (including these concentrations), which follows linearity and has a suitable level of precision and accuracy.

- **Accuracy (trueness)** is the closeness between an accepted true or reference value and measured value, in an analytical method. This is sometimes also termed as trueness of the method.

- **Recovery** describes the amount of an analyte, in the sample after sample preparation steps (Preconcentration/extraction). It also expresses the sampling efficiency.

- **Precision** represents the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same
homogeneous sample under prescribed conditions. It is expressed as a relative standard deviation (RSD), which is obtained by dividing the standard deviation (SD) with the mean of the analyte value (concentration or amount).

Precision can be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability (intra-assay precision) measures the variation of multiple determinations of a single sample under the same operating conditions over a short interval of time, whereas intermediate precision (inter-assay precision) expresses variations within laboratories, i.e. different analysts, different days, different equipment, etc. It is not considered necessary to study these effects individually, always. Reproducibility, however, measures the precision between laboratories and it is usually measured in collaborative studies usually applied to standardization of methodology.

- **Limit of detection (LOD)** is the lowest amount of an analyte in a sample that can be distinguished from the background i.e. detected, but not necessarily quantified as an exact value. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. Based on signal-to noise (S/N) method, LOD is the injected amount that results in a peak with a height three times as high as the baseline noise level i.e. S/N=3.

- **Limit of quantification (LOQ)** is the lowest amount of an analyte in a sample, which can be quantified with acceptable precision and accuracy. For chromatographic methods the LOQ can also be determined through comparing measured signals from samples with known low concentrations of analyte with those of blank samples with a typical signal-to noise ratio (S/N) of 10:1.

- **Robustness** measures the capacity of an analytical procedure to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of the reliability of the method under normal usage. The parameters tested within robustness typically include the stability and quality of the sample and analytical solutions i.e. pH, a number of method parameters, such as mobile phase, flow rate, column temperature, injection volume, detection wavelength, are varied within a realistic range, and the quantitative influence of the variables is determined. One consequence of robustness is that
a series of system suitable (optimized) parameters is established to ensure the validity of analytical method whenever used.

The extent and requirements for the analytical method validation depend on the developmental phase of the method and the sample matrix for which the method is applied. The validation procedure should be defined already at the beginning of the method development. The validation parameters should be selected based on the nature and purpose of the method. The validation process, should serve the researcher as a analytical tool to provide complete understanding on the characteristics and the performance of the method.

In the final, the purpose of validating methods is to ensure the procurement of high quality data and if the quality of data is questionable, no meaningful conclusions can be reached about the authenticity of the developed analytical method and demonstration of method validity also relies on the responsibility of the researcher involved in the development of the method. Perhaps the single most important element required is a good understanding of what validation is. This understanding actually goes beyond the concept of requiring a minimum of three or five runs. This understanding must be anchored by sufficient, practical knowledge and experience. On analytical regulatory platform, time invested in validating analytical methods pays a big dividend in the long run.

1.4.7 Important analytical terms

**Analyte:** A specific chemical moiety being measured in a given matrix (environmental, biological and/or food).

**Analytical run (or batch):** A complete set of analytical and study samples with appropriate number of standards and QCs for their validation. Several runs (or batches) may be completed in one day, or one run (or batch) may take several days to complete.

**Matrix:** A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, faeces, saliva, sputum, and various discrete tissues.

**Calibration standard:** A biological matrix to which a known amount of analyte has been added or spiked. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.
**Matrix effect:** The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

**Method:** A comprehensive description of all procedures used in sample analysis.

**Quantification range:** The range of concentration, including ULOQ and LLOQ, that can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration-response relationship.

**Sample:** A generic term encompassing controls, blanks, unknowns, and processed samples, as described below:

**Blank:** A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

**Quality control sample (QC):** A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

**Unknown:** A biological sample that is the subject of the analysis.

**Standard curve:** The relationship between the experimental response value and the analytical concentration (also called a calibration curve).

**Validation:** Establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.

### 1.5 RATIONALE

“My Ph.D. thesis topic can be approached from a variety of points of view, but I adopt the perspective of an analytical chemist. Because of the interdisciplinary character of the topic, I realise that the topic has a broad spectrum of backgrounds, and, however, I confine it to the development of novel analytical methods and their environmental applications.”

In recent time, debate has peaked about toxic vignette of the chemicals in the global environment. More than 800 studies\(^\text{14}\) concluded that it is “remarkably common” for these toxic chemicals to induce biological responses at much lower doses than expected, and for the responses to be non-monotonic. The assertion has huge regulatory implications because safety testing for most chemicals is done not at the low concentrations (doses) at which agents may occur in the environment, but at high doses. The results are extrapolated to lower doses, a methodology that would be unsound if
non-monotonic behaviour were widespread. With little data on how many, and how much toxic chemicals are in the drinking water and source waters, soil, environment and consumer products and even less showing a cause-effect relationship between an active ingredient and an adverse effect, researchers, health and environmental agencies, and water-quality regulators have been playing hot potato with the question for decades.

‘Concurrent changes and advances in analytical methods will set the groundwork for how we view chemical safety for decades to come.’ The advance, give us unprecedented opportunity to apply these, to determine and understand the environmental fate of many toxic chemicals, at trace levels. These novel analytical protocols will provide an opportunity to ‘improve upon the regulatory construct’ by giving the regulatory agencies more cost-effective analytical tools to make the determinations about toxic chemicals.

In the present study, novel analytical protocols well versed in environmental and health topics, or productive in developing methods that lighten the environmental and health footprints, has been developed, and would be key players in the development of the new green world.

The key contents of the rationale are elucidated as following:

1.5.1 Choice of target compounds and water as a sample matrix

The studied toxic compounds are EPA priority pollutants (MNP, PAH, DEHP), chemical of concern (BPA) and/or endocrine disruptors (Estrogens), abound in residential and source waters, with high public concern, high production volume, toxicological significance and high potential for human exposure from use and occurrence in the environment. Besides, the availability of economical analytical standards and chemical reagents, feasibility of HPLC-UV/FLD analysis is deliberately considered.

Water touches countless day-to-day activities, during which humans get exposed to inherent toxic substances. This focus on exposure essentially regards the reality that toxic substances produce health problems only if they reach the body and, water is the easy and highly accessed medium of exposure to these studied toxic substances. This rationalise the applications of the innovated analytical methods to the various water samples including bottled water, drinking water and source waters.
1.5.2 Contribution to water safety, security and sustainability

Provision of toxic free water for human drinking, domestic and other uses can be viewed as a fundamental example of water security and sustainability: survival is impossible without consuming water in some form, but sufficient water for survival alone is far from adequate, for a tolerable or healthy life. One of the most pervasive problems afflicting humans throughout the world is inadequate access to toxic free clean water. Addressing this problem calls out for a tremendous amount of research to be conducted to innovate robust new methods of water analysis and purification at lower cost and with less energy, while at the same time minimizing the use of chemicals and impact on the environment. Here, in this study, we highlight some of the novel analytical methods being developed to sense and quantify low-concentration toxic contaminants in water (bottled water, drinking and source waters) at lower cost, without intensive use of chemicals or production of toxic byproducts, all of which hold great promise for effectively improve quality water supplies.

1.5.3 Contribution to the society: Today’s society is beautiful, but is a toxic world to live in. Cataloguing the potential risks that are posed to society by toxic substances, has resulted in a perceived need for action and hence a requirement for ‘usable analytical science’. To address this, this study is an important piece of research work, that provides a window into ultra-trace concentrations of toxics, and the developed novel analytical protocols represent 'a leap forward' in our view of the grim scenario of toxics in today society.

1.5.4 Contribution to the knowledge / subject: To detect ultra-trace quantities of toxics, remains a challenging problem in analytical chemistry. The tour de force of this study is the potential gain in sensitivity that can be obtained when employing the proposed analytical methods. From concepts to practice, from table to bench, this research work presents detailed, ready-to-use analytical protocols, which were developed to determine concentrations of toxics upto the level of pg/mL and fg/mL, in environmental and drinking waters. This includes novel analytical methods for the determination of Bisphenol A (pg/mL), Nitrophenols (ng/mL), Di-2-ethyl hexyl phthalate (pg/mL), estrogens (pg/mL) and polyaromatic hydrocarbons (fg/mL) respectively, in drinking and environmental waters. All the methods are reliable, reproducible, adhere to ‘Good Laboratory Practice’ standards and cover all the required steps from sampling to the interpretation of the results including data on precision, accuracy, detection limit, and calibration.
1.5.5 Contribution to green chemistry

Green chemistry is the today’s buzz. This study innovates green analytical protocols for the analysis of toxics, concentrating on minimizing sample preparation and handling volumes, reducing reagent and solvent consumption, minimizing of waste and toxic byproducts, operator safety and economic savings, while preserving the classic analytical parameters of accuracy, sensitivity, selectivity, and precision. Thus, this study is a green refill to the chemistry.

1.6 APPLICATIONS OF THE STUDIES

The analytical study is capable of detecting and quantitating the targeted (studied) toxics, at trace and ultra-trace levels, with an eye for practical applications, in real water matrices.

Targeted toxics are:

- **Bisphenol A**
- **Mononitrophenols**
- **Estrogens**
- **Polyaromatic hydrocarbons**
- **Di-2-ethylhexyl phthalate**

The key potential applications of the study may be as following:

1. In terms of analysis, we developed very effective and sensitive protocols to quantify a broad spectrum of toxics.
2. Increased sensitivity allows us to investigate toxics, at trace levels, have continuous exposure, and are toxic.
3. Confirmation of toxicants for the establishment of cause-effect relationship studies in toxicology.
4. The hazardous potential of toxic substances in environmental water samples can be assessed.
5. Drinking and source water quality monitoring.
7. Environmental water protection.

8. Leads the way for analytical chemists developing new analytical protocols for different toxics. The overall analytical approach in this study can be applied (allow easy expansion) to other phthalates, nitrophenols, estrogens, PAHs and indeed other contaminants with similar properties (related compounds like octyl and nonyl phenol, bisphenol S, bisphenol F), and applications can be expanded to biological samples also (blood, urine) and can assist in ensuring the safety of cosmetics, environment, food, and water.

9. In cooperation with other disciplines, we may apply these analytical protocols to improve our understanding of the occurrence and behaviour of trace contaminants in different matrices and to better understand biological effects.

1.7 CONCLUSION

In today’s scenario, analysis of toxics underpins almost all aspects of our daily life and has a high profile related to human health and safety, environmental pathways and monitoring, risk assessing investigations, toxicology, the protection of commercial interests and contingency responses. This piece of research work focuses on research and development of analytical methods that are intended to serve as a basis for future innovations and approaches to analytical solutions (trace level determination) of toxic vignette of the environment. Analytical studies in this research cover a wide spectrum of toxic substances, and innovate application-orientated analytical methods. In our pursuit to provide trace level analytical ground for scientific research with state-of-the-art quality, this study will serve as a valuable resource to meet analytical needs in toxic science, such as:

• Broaden analytical scope

• Lower detection limits

• Increase selectivity

• Better trueness and precision

• Improve confidence in identifications
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